From DEPARTMENT OF BIOSCIENCES AND NUTRITION
Karolinska Institutet, Stockholm, Sweden

# EXPLORING THE GENOME-WIDE IMPACT OF TRANSCRIPTION FACTOR AP-1 IN BREAST CANCER

Yichun Qiao


Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by E-Print AB 2015

ISBN 978-91-7676-142-7

# EXPLORING THE GENOME-WIDE IMPACT OF TRANSCRIPTION FACTOR AP-1 IN BREAST CANCER

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Yichun Qiao**

*Principal Supervisor:*
Docent Chunyan Zhao
Karolinska Institutet
Department of Biosciences and Nutrition

*Co-supervisor:*
Professor Karin Dahlman-Wright
Karolinska Institutet
Department of Biosciences and Nutrition

*Opponent:*
Professor Olle Stål
Linköpings universitet
Department of Clinical and Experimental Medicine
Division of Clinical Sciences

*Examination Board:*
Professor Anki Östlund-Farrants
Stockholms universitet
Department of Molecular Biosciences

Docent Johan Hartman
Karolinska Institutet
Department of Oncology and Pathology

Professor Peter Zaphiropoulos
Karolinska Institutet
Department of Biosciences and Nutrition

*To my dearest family*

# ABSTRACT

AP-1 plays crucial roles in a wide range of cellular processes in breast cancer. Through the dimeric basic leucine zipper (bZIP) domain, the mammalian AP-1 proteins bind to DNA and form homodimers or heterodimers from the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2), ATF and MAF family members. AP-1 is involved in several signal transduction pathways to control physiological and pathological processes, such as oncogenesis, metastasis and apoptosis. However, the mechanistic aspects of the modulatory effect of AP-1 in breast cancer are still not fully understood. Thus, to explore genome-wide transcriptional regulatory networks of the transcription factor AP-1 in breast cancer may help to identify novel strategies to develop new therapies.

In **Paper I**, we established that AP-1 participates in estrogen-dependent gene expression and proliferation programs in breast cancer cells. In addition, we identified PKIB (cAMP-dependent protein kinase inhibitor-β) as a novel ERα/AP-1 target molecule, which is required for breast cancer cell growth.

In **Paper II**, we observed, by analyzing publically available datasets, that AP-1 is expressed at high levels in basal-like breast cancers and associated with poor clinical outcome. High level expression of AP-1 was also found in triple-negative breast cancer (TNBC) cell lines as determined by Western blot analysis and qPCR. Using cistrome and transcriptome analyses to investigate the signaling networks of AP-1 in TNBC cells, we identified that about 15% of AP-1 binding sites are located in the proximal 5' region of the nearest gene. Gene expression profiling analysis identified differential expression of 419 and 690 genes upon knockdown of Fra-1 and c-Jun, respectively. Among these genes, 222 genes which were regulated by both Fra-1 and c-Jun were associated with cytokine-mediated signaling, type I interferon-mediated signaling, chemotaxis, cell adhesion, immune response, cell junction assembly, adherens junction organization and inflammatory response. Moreover, we found that proliferative phenotypes of TNBC cells were inhibited upon depletion of AP-1. In addition, silencing of AP-1 reduced the invasion ability both *in vitro* and *in vivo*. We further showed that AP-1 activation, downstream of the PI3K/Akt and MAPK/ERK pathways, repressed expression of E-cadherin by transcriptional upregulation of ZEB2.

In **Paper III**, we demonstrated that TNFα activated both the PI3K/Akt and MAPK/ERK signaling pathways to induce epithelial-mesenchymal transition (EMT) in TNBC cells via activation of AP-1 signaling and increased expression of the EMT regulator ZEB2. Based on published data on spliced transcripts, two alternatively spliced 5'UTR isoforms of the ZEB2 gene were found to be expressed in breast cancer cell lines and breast tumor samples. Using the chromosome conformation capture assay, we demonstrated that AP-1, when activated by TNFα bound to a site in promoter 1b of the ZEB2 gene where it regulates the expression of both promoter 1b and 1a, the latter via mediating long range chromatin interactions.

In **Paper IV**, We defined that c-Jun regulated nearly a third of the TNFα-elicited transcriptome. Expression of a c-Jun-regulated pro-invasion gene set was shown to be strongly associated with clinical outcomes in TNBCs. We demonstrated that c-Jun drives TNFα-mediated TNBC malignant characteristics by transcriptional regulation of Ninj1. As exemplified by the c-Jun bound CXC chemokine genes clustered on chromosome 4, we demonstrated that NF-κB might be a pioneer factor and was required for the regulation of TNFα-inducible inflammatory genes, whereas c-Jun had little effect on TNFα-inducible inflammatory genes.

In conclusion, our studies give additional insights into the molecular mechanisms of AP-1 in relation to breast cancer cellular processes. We suggest that inhibition of AP-1 could be a new therapeutic strategy for treatment of breast cancer, especially TNBC.

# LIST OF SCIENTIFIC PAPERS

I. Dahlman-Wright K, **Qiao Y**, Jonsson P, Gustafsson JÅ, Williams C, Zhao C. *Interplay between AP-1 and estrogen receptor α in regulating gene expression and proliferation networks in breast cancer cells.* Carcinogenesis. 2012 Sep;33(9):1684-91

II. Zhao C*, **Qiao Y***, Jonsson P, Wang J, Xu L, Rouhi P, Sinha I, Cao Y, Williams C, Dahlman-Wright K. *Genome-wide Profiling of AP-1-Regulated Transcription Provides Insights into the Invasiveness of Triple-Negative Breast Cancer.* Cancer Res. 2014 Jul 15;74(14):3983-94

III. **Qiao Y**, Shiue C, Zhu J, Jonsson P, Zhuang T, Williams C, Wright A, Zhao C, and Dahlman-Wright K. *AP-1-mediated chromatin looping regulates ZEB2 transcription: new insights into TNFα-induced epithelial-mesenchymal transition in triple-negative breast cancer*. Oncotarget. 2015 Apr 10;6(10):7804-14

IV. **Qiao Y**, Jonsson P, Indranil S, Zhao C, and Dahlman-Wright K. *AP-1 is a key regulator of TNFalpha-mediated triple-negative breast cancer progression*. Manuscript

* Both authors contributed equally to the work

# Related paper (not included in this thesis)

Zhu J, Zhao C, Kharman-Biz A, Zhuang T, Jonsson P, Liang N, Williams C, Lin CY, **Qiao Y**, Zendehdel K, Strömblad S, Treuter E, Dahlman-Wright K.*The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor α and modulates estrogen-stimulated breast cancer cell proliferation.* Oncogene. 2014 Aug 21;33(34):4340-51. doi: 10.1038/onc.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3C | Chromosome confirmation capture |
| Akt | v-akt murine thymoma viral oncogene homolog |
| ATF | Activating transcription factor |
| AP-1 | Activator protein 1 |
| BrdU | Bromodeoxyuridine-labeling experiments |
| bZIP | Basic leucine zipper |
| ChIP | Chromatin immunoprecipitation |
| ChIP-seq | Chromatin immunoprecipitation followed by sequencing |
| CLCA2 | Chloride channel accessory 2 |
| CRE | Cyclic AMP responsive elements |
| E2 | 17-β Estradiol |
| E2F1 | E2F transcription factor 1 |
| EMT | Epithelial-to-Mesenchymal Transition |
| ER | Estrogen receptor |
| ERK | Extracellular signal-regulated kinase |
| ERα | Estrogen receptor α |
| GO | Gene ontology |
| HER2 | Human epidermal growth factor receptor2 |
| IKK | IκB kinases |
| JNK | Jun amino-terminal kinase |
| MAPK | Mitogen-activated protein kinase |
| MMP9 | Matrix metallopeptidase 9 |
| NF-κB | Nuclear factor kappa-light chain enhancer of activated B-cells |
| Ninj1 | Ninjurin 1 |
| PI3K | Phosphoinositide-3 kinase |
| PKIB | Protein kinase (cAMP-dependent, catalytic) inhibitor beta |
| PR | Progesterone receptor |
| RIP | Receptor-interacting protein |
| STAT6 | Signal transducer and activator of transcription 6 |

| | |
|---|---|
| siRNA | Small interfering RNA |
| TNBC | Triple negative breast cancer |
| TNFα | Tumor necrosis factor α |
| TNFR1 | Tumor necrosis factor receptor 1 |
| TNFR2 | Tumor necrosis factor receptor 2 |
| TPA | 12-*O*-tetradecanoylphorbol-13-acetate |
| TRAF2 | TNF receptor-associated factor 2 |
| TRE | TPA response element |
| TSS | Transcription start site |
| VEGF | Vascular endothelial growth factor |
| ZEB2 | Zinc finger E-box binding homeobox 2 |

# 1 INTRODUCTION

## 1.1 Activator Protein-1

AP-1 (activator protein-1) as a transcription factor is a dimeric complex. It was originally characterized in early 1987 (*1*). Mammalian AP-1 proteins are composed of multiple members of the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1 and Fra2), ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) and MAF (c-Maf, MafA, MafB, MafG/F/K and Nrl) subfamilies (*2*, *3*).

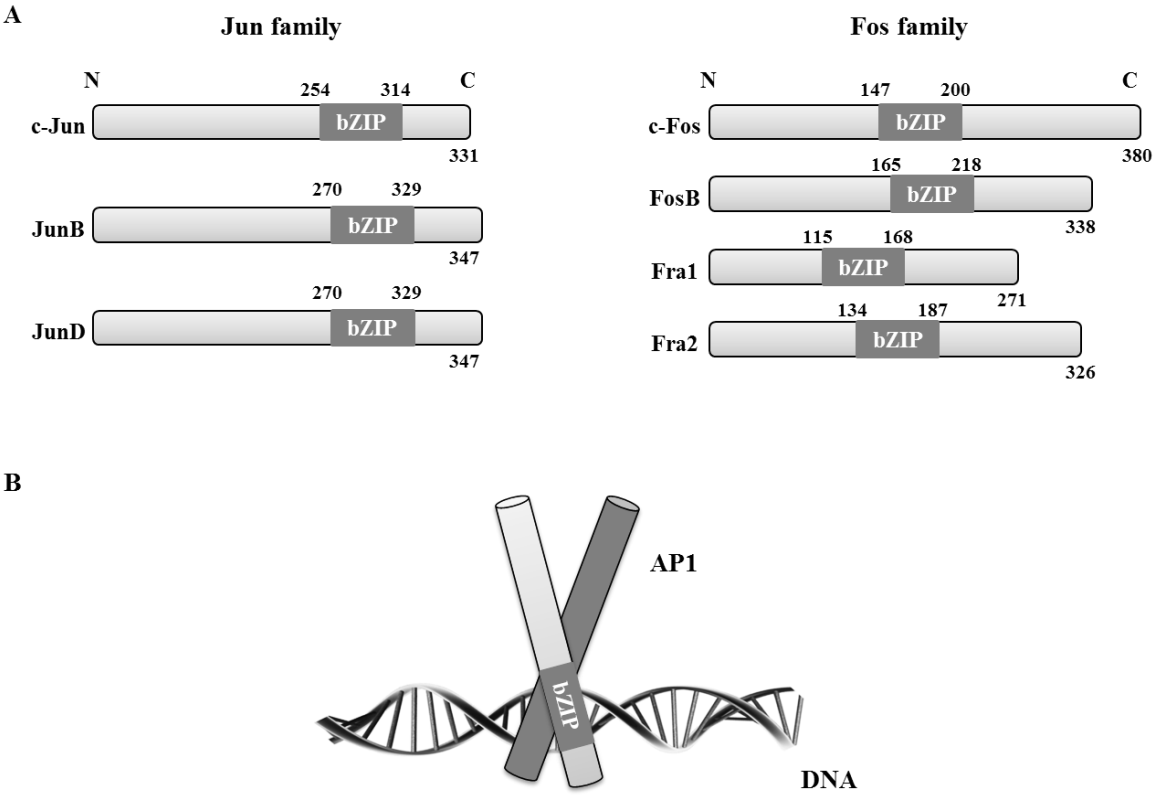


**Figure 1. AP-1 structure.** (A) The location of bZIP domain in Jun and Fos family members. (B) The AP-1 complex binds to DNA as homo- or heterodimers through the bZIP domain. Numbers represent amino acids from the amino to the carboxy termini.

AP-1 proteins dimerize and bind to DNA through the bZIP (basic leucine zipper) DNA binding domain which is composed of leucine zipper and basic regions (*4, 5*) (Figure 1). In the Jun family, due to a lower degree of conservation of the amino-terminal region from amino acids 1 to 95, 3 members of Jun protein are described although they share a high degree of sequence homology (*6, 7*). Compared to homodimerized Jun proteins, Fos-Jun heterodimers form more stable dimers when binding to TPA (12-*O*-tetradecanoylhporbol-13-acetate) response elements (TRE, 5'-TGAC/GTCA-3'). Fos family members do not homodimerize (*8, 9*). Unlike Jun-Jun and Jun-Fos dimers, the Jun-ATF heterodimer bind to another consensus sequence, the cAMP-responsive element (CRE, 5'-TGACGTCA-3') (*10*).

Various Fos-Jun dimers are present not only in different cell types under different conditions, but also in different cell cycle stages (*11-13*). For example, after serum stimulation in mouse fibroblasts, initially c-Fos and FosB form heterodimers with c-Jun and JunB during the $G_0$-to-$G_1$ transition , then Fra1 and Fra2 are the predominant Fos proteins to form heterodimers with all members of Jun proteins during exponential growth (*12*). It has been reported that more than 50 different proteins interact with Fos-Jun family members and contribute to AP-1 functions (*14*). As shown in Figure 2, there are three different models of other proteins interacting with Fos-Jun proteins, 1) by bZIP domain, such as, NF-E2 (Nuclear Factor, Erythroid 2) family members (*15*); 2) by binding to regulatory elements adjacent to AP-1 sites (TGAGTCA) directly, such as NFAT (Nuclear factor of activated T-cells) protein; 3) by binding to regulatory elements adjacent to AP-1 sites indirectly, such as Smad family members which need to interact with other factors to bind the DNA (*16, 17*).

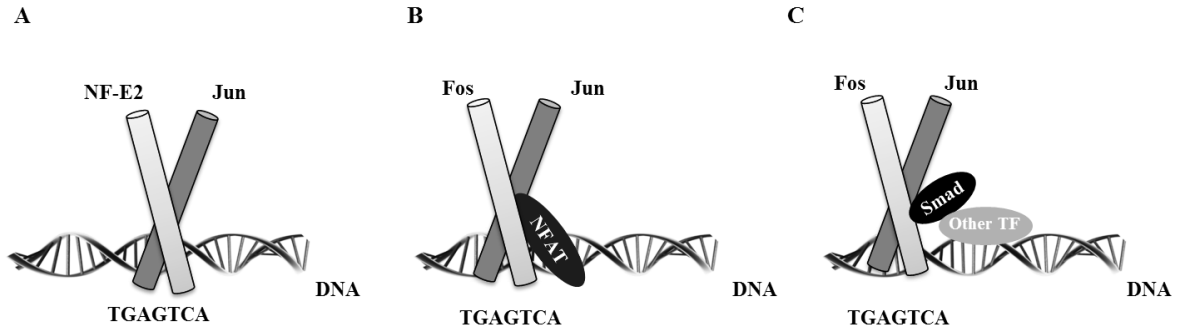


**Figure 2. Models of Fos-Jun interactions with other proteins.** A. Via the bZIP domain. B. By direct binding to regulatory elements adjacent to AP-1 sites. C. By indirect binding to regulatory elements adjacent to AP-1 sites. TGAGTCA: AP-1 site.

### 1.1.1 Jun family

The Jun family consists of c-Jun, JunB and JunD. The JunB and JunD genes are located on chromosome 19, and the c-Jun gene is located on chromosome 1. It was reported that Jun family members are highly expressed and play an important role in different tumor types such as non-small-cell lung cancer, prostate cancer, breast cancer, colorectal adenocarcinoma and acute myeloid leukemia (*18-22*). It is well known that c-Jun can promote cell proliferation, survival and apoptosis. In contrast, JunB and JunD have been shown to act as tumor repressors in some studies (*23-25*).

N-terminal phosphorylation of c-Jun is crucial for its activation and is triggered by post-translational modifications mainly controlled by JNKs (Jun N-terminal kinases), such as ERK (extracellular-signal-regulated kinase) and p38 isoforms (*7*). JNK phosphorylates Ser63, Ser73, Thr91 and Thr93 of the c-Jun protein, increasing the stability, DNA binding and transactivation potential of c-Jun (Figure 3). Other transcription factors such as SP1 (specificity protein 1), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MEF2 (myocyte enhancer factor-2) can induce the transcription of c-Jun and increase its mRNA stability resulting in increased levels of c-Jun (*26*). Subsequently, c-Jun induces rapid stimulation of genes involved in promoting the cell cycle such as cyclin D1 or repression of negative regulators of the cell cycle such as the tumor suppressor p53 (*27, 28*).

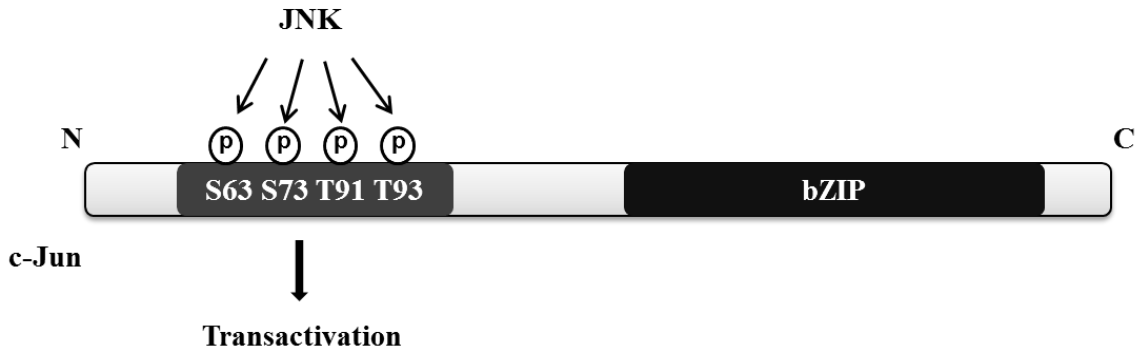


**Figure 3. c-Jun protein domain structure and phosphorylation sites.** S, serine. T, threonine.

Unlike c-Jun, JunB lacks phosphor-acceptor residues and JunD lacks an effective JNK docking site and subsequently they are weak phosphorylation substrates of JNK. However,

MAPK/ERK can phosphorylate JunD, making the transcriptional activity of JunD stronger than that of JunB (*29*). Normally, JunB and JunD are considered as tumor suppressor genes (*23, 30*). However, in the absence of c-Jun, JunB has been demonstrated to exhibit proliferative effects by inhibiting the expression of p53 in mice (*31*).

### 1.1.2 Fos family

The Fos family consists of c-Fos, FosB, Fra1 and Fra2, the corresponding genes of which are located on chromosome 14, chromosome 19, chromosome 11 and chromosome 2, respectively. c-Fos and FosB harbor a strong transactivation domain in their C-terminal part, which Fra1 and Fra2 do not have (*32*). Subsequently, Fra1 and Fra2 transactivation potencies are weaker than the corresponding activities of c-Fos and FosB. In contrast to Jun family members which can form homodimers, Fos family members have to form heterodimers with Jun or ATF family members to regulate gene expression.

Fos proteins are widely expressed in various tumors, including bone tumors (*33, 34*), endometrial carcinoma (*35, 36*), cervical cancer (*37, 38*), ovarian cancer (*39, 40*), mesotheliomas (*41, 42*), lung cancer (*43, 44*), colorectal cancer (*45, 46*), skin tumors (*47, 48*), melanomas (*49, 50*), thyroid carcinomas (*51, 52*), esophageal cancer (*53, 54*), hepatocellular carcinomas (*55, 56*) and breast cancer (*57, 58*). As shown in Figure 4, the activation of c-Fos can occur due to phosphorylation by ERK at T325, T331 and S374, also by RSK1 (ribosomal s6 kinase1) and RSK2 at S362 (*59*). In addition, ultraviolet light can activate c-Fos by phosphorylation at T232, T325 and T331 via p38/MAPK kinases (*60*). Stabilization of c-Fos relies on ERK-mediated phosphorylation, while Fra1 stabilization also relies on ERK-mediated phosphorylation but at different sites (*59*).

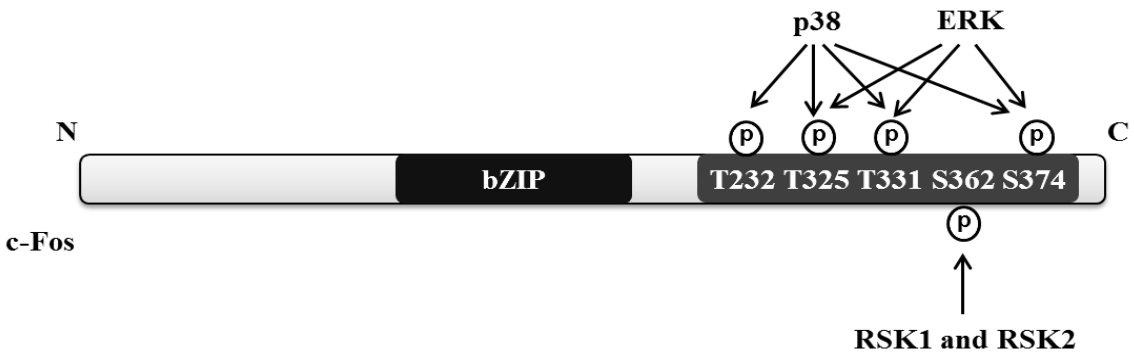


**Figure 4. c-Fos protein domain structure and phosphorylation sites.**

### 1.1.3 AP-1 signaling pathways

AP-1 activity is induced by complex networks of signaling pathways that involve several physiological stimuli and environmental insults, such as growth factors, cytokines, UV irradiation and viral infections (*61*, *62*).

As shown in Figure 5, growth factor stimulation activates PI3K/AKT and ERK, the latter which is the subgroup of MAPKs pathways that enhance AP-1 activity (*63*). The ERK subgroup can directly phosphorylate Fra1 and Fra2 and enhance their DNA binding activity when in complex with c-Jun (*64*). In addition, Fra1 and c-Jun are mainly induced by activated Ras signaling pathways compared to other AP-1 family members (*65*). TNFα and cytokines potently induce AP-1 through activating JNK signaling which can phosphorylate AP-1 and thereby enhance its transcriptional activity (*66*). Once AP-1 is activated, it is able to regulate its targeted genes, leading to regulation of proliferation, apoptosis, inflammation, invasion and EMT (Epithelial-to-Mesenchymal Transition).

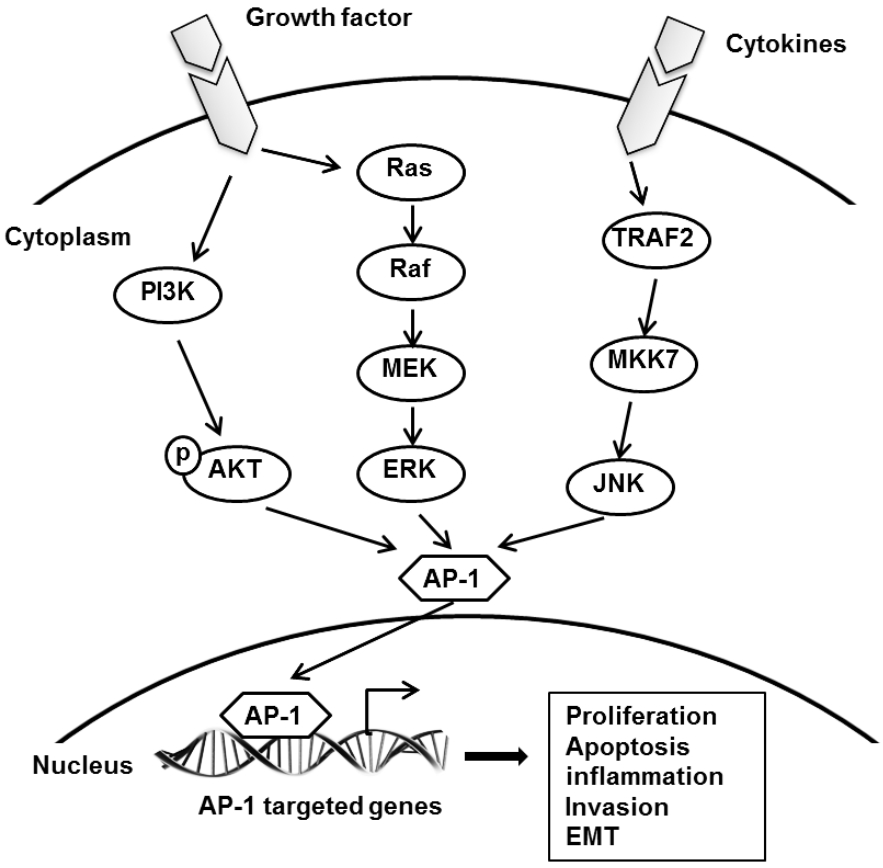


**Figure 5. Signaling pathways up-stream of AP-1**

## 1.2 AP-1 function

AP-1 plays an important role in a wide range of physiological and pathological cellular processes from cell proliferation and development to invasion, apoptosis and EMT. Although all AP-1 members contain the bZIP domain, the individual proteins have different effects in different kinds of cell types. These diverse functions are due to differential dimerization of AP-1 family members which alter the promoter binding, transcriptional capacity, protein stability and localization of AP-1 complexes (*67-69*).

The AP-1 transcription complex is often considered as an oncoprotein (*1*). However, whether AP-1 is oncogenic or anti-oncogenic not only depends on the differential dimerization between AP-1 family members, but also depends on the tumor types, tumor stages and the genetic background of tumors. For example, it has been shown that JunB acts as a tumor suppressor for development of chronic myeloid leukemia (*70*). In contrast, JunB promotes cell invasion and angiogenesis in VHL (von Hippel-Lindau) -defective clear-cell renal-cell carcinoma (*71*).

### 1.2.1 Cell proliferation

Among AP-1 family members, c-Jun has been reported in several studies that it acts primarily as a positive regulator of cancer cell proliferation, such as in classical Hodgkin lymphoma, glioblastoma and breast cancer (*72-74*). c-Jun protein has been demonstrated to be activated by phosphorylation via JNK signaling, inhibiting the expression of p53 or inducing cyclin D1 expression to promote cell proliferation (*75, 76*). In addition, c-Jun is required for liver regeneration *in vitro* (*28*).

Unlike c-Jun, JunB and JunD attribute mainly to anti-proliferative effects in most studied systems. JunD inhibits the cell proliferation of intestinal epithelial cells and fibroblasts (*30, 77*). Overexpression of JunB inhibits malignant skin cell proliferation *in vivo* and *in vitro* (*78*). However, it has been described that not only JunB and JunD, but also c-Jun has anti-proliferative function, such as in fibroblasts and multiple myeloma (*79, 80*). c-Jun has been shown to exert also an anti-proliferative function in osteoclasts when dimerized with c-Fos (*81*). Additionally, the expression of JunB and JunD may be positively correlated with cell proliferation in diffuse large B-cell lymphomas (*82*).

For the Fos family, high expression of FosB is negatively correlated with p16 which is a tumor suppressor protein. This might contribute to an important role of FosB in regulating normal proliferation and differentiation of mammary epithelial cells (*83*). Inhibiting the expression of Fra1 induces a tumor supportive set of secreted signals in melanoma cells *in vivo* and *in vitro* (*84*). c-Fos contributes to promote cell proliferation through ERK1/2 signaling pathways in primary rat hepatic stellate cells (*85*). Fra2 can stimulate cell growth by forming heterodimers with JunD in adult T-cell leukemia cells (*86*).

Other AP-1 family members also have their pivotal roles in cell proliferation. c-Maf can promote myeloma proliferation by activating the cyclin D2 promoter (*87*). For the ATF family, ATF-2 can activate cell proliferation (*88*), whereas another ATF family member, B-ATF, is considered as a tumor suppressor gene (*89*).

### 1.2.2 Apoptosis and survival

The pro-apoptotic or anti-apoptotic functions of AP-1 are not only dependent on specific cell type, but are also dependent on heterodimerization between different AP-1 members and the type of external or internal stimuli.

Overexpression of c-Jun has been shown to induce apoptosis in multiple myeloma cells, esophageal adenocarcinoma and endothelial cells (*79, 90, 91*). c-Jun and c-Fos are required to promote inflammation and cell death in skin tissue (*92*). c-Fos also has a pro-apoptotic function in prostate cancer cells upon TNF–related apoptosis-inducing ligand (TRAIL) stimulation (*93*). However, under cisplatin treatment, c-Fos plays an anti-apoptotic role in thyroid cancer (*94*). Combination of ATF3 and c-Jun expression can promote survival in neurons during injury (*95*).

### 1.2.3 Invasion and metastasis

EMT is one of the hallmarks of invasion and metastasis. It is a process by which epithelial cells lose their cell polarity and cell-cell adhesion to become mesenchymal stem cells. Extensive evidence suggests that AP-1 may be a key mediator to regulate gene expression in relation to EMT. For example, AP-1 directly regulates invasion effector genes, such as MMP9 (matrix metallopeptidase 9) and CD44, or invasion suppressor genes, such as fibronectin and

STAT6 (signal transducer and activator of transcription 6) *in vitro* and *in vivo*. In addition, AP-1 can regulate cell invasion by interacting with other transcription factors, such as NFκB and Ets in a variety of human tumor cells (*96*).

In recent years, it has been reported that c-Jun and Fos proteins contribute to TGF-β1-induced EMT in breast cancer, but not JunD and FosB (*97, 98*). Mitochondrial dysfunction which induces EMT is dependent on c-Jun activation (*99*). In colorectal cancers, c-Jun and ATF-2 but not c-Fos are required for Twist1-induced EMT by phosphorylation via JNK (*100*).

### 1.2.4 Angiogenesis

A direct role for c-Jun in angiogenesis through VEGF (vascular endothelial growth factor)-induced neovascularization was identified in rodents in 2004 (*101*). Later, a correlation between c-Jun activation and angiogenesis was confirmed in breast cancer patients (*72*). JunB promotes angiogenesis through regulating the expression of MMP9 and MMP2 in VHL (Von Hippel-Lindau)-defective renal cell carcinoma (*71*). In addition, increased c-Fos and c-Jun heterodimer formation and DNA binding lead to lymphangiogenesis upon IL7 (Interleukin 7) stimulation in lung cancer (*102*). The expression of Fra1 is also necessary for angiogenesis *in vitro* and *in vivo* (*103*).

### 1.2.5 AP-1 in mouse development

Different Jun and Fos knock-out mice models exhibit various phenotypes (*104*). Fra1, c-Jun or JunB knock-out in mice present with an embryonic lethality phenotype and mice die around day 10 (*105-107*). Mice lacking Fra1 have defects in the placenta and the yolk sac, while mice lacking c-Jun have defects in the heart and the liver. Lack of JunB not only causes defects in the placenta and the yolk sac, but also vascular defects in extra-embryonic tissues. Mice lacking c-Fos, FosB, Fra2 or JunD can develop normally. However, inactivation of c-Fos leads to osteopetrosis and the null c-Fos mice have more wakefulness (*108, 109*). Inactivation of FosB leads to nurturing defects due to changes in the hypothalamus region (*110*). Inactivation of JunD leads to sterility and age-related endothelial dysfunction in male. Inactivation of Fra2 leads to defects in chondrocytes and osteoclasts in newborn mice (*104, 111, 112*).

## 1.3 Breast cancer

Breast cancer is the most common female cancer worldwide, and the incidence is about 100 times higher in women compared to men. Of all types of cancer, 25% of them are breast cancer, and breast cancer is strongly related to age. In African-American women, breast cancer is more common in women who are under 45 years of age and they have a higher risk to die. Some risk factors for breast cancer have been identified, such as genetic predisposition (particularly BRCA1 and BRCA2 mutations), family history, taking hormones and older age at first birth or never having given birth. However, most of the patients do not present with specific risk factors. In the whole world, 21% of deaths in breast cancer are associated with obesity, high intake of alcoholic beverages and lack of physical activity (*113-116*).

### 1.3.1 Breast cancer molecular subtypes

Breast cancer can be classified according to histopathology, stage and histological grade. However, these classifications do not give detailed biological characteristics of the tumors that have similar clinical and pathological presentations. Therefore, global gene expression microarray studies that classify breast cancer into distinct biological classes based on gene expression patterns have opened a broad field in cancer research regarding breast cancer initiation, development and identification of potential new therapy targets.

Gene expression profiles classify breast cancer into five intrinsic molecular subtypes, including luminal A and B, HER2-positive, basal-like and normal breast-like (*117*). Immunohistochemical (IHC) staining classifies the breast cancer into three molecular subtypes: ER-positive, HER2-positive and triple-negative. Clinically, substantial numbers of TNBC (triple-negative breast cancer) are basal-like subtype. However, the discordance between the gene-based and immunohistochemical-based profiles is considerable (*118, 119*) (Table 1).

**Table 1. Molecular subtypes of breast cancer**

| Intrinsic subtypes (%) | | IHC | ER | PR | HER2 | Other markers | Therapy |
|---|---|---|---|---|---|---|---|
| Luminal (75%) | Luminal-A (50%-60%) | Luminal-A | + | + | - | Low Ki67 | ➢ Endocrine therapy for the majority of cases<br>➢ Chemotherapy is considered in case of high tumor burden or grade 3 |
| | Luminal-B (15%-20%) | Luminal-B | + | +/- | +/- | ➢ High Ki67<br>➢ High expression of proliferation-related genes compared with Luminal-A | ➢ Endocrine therapy + Chemotherapy for the majority of cases<br>➢ Endocrine therapy + Chemotherapy + Anti-HER2 for HER2-positive |
| HER2-positive (15%-20%) | | HER2-positive | - | - | + | Other genes associated with the HER2 pathway | Chemotherapy + Anti-HER2 |
| Basal-like | Classic basal-like (8%-37%) | Triple-negative | - | - | - | ➢ High levels of basal myoepithelial markers<br>➢ Overexpress P-cadherin,fascin,caveolins1 and 2, alpha-beta crystalline and EGFR | Chemotherapy |
| | Claudin-low (7%-14%) | Triple-negative | - | - | - | ➢ Low expression of claudins 3,4 and 7<br>➢ Enrichment for EMT markers | Chemotherapy |
| Normal breast-like (5%-10%) | | Triple-negative | - | - | - | ➢ CK5-<br>➢ EGFR- | Chemotherapy |

Approximately 75% of breast cancers are of the luminal subtype which can be further divided into luminal A and luminal B based on expression of Ki67 (*120*). Luminal A is the most common subtype. Patients with luminal A have good outcomes with hormonal therapy, and have low expression of Ki67 compared with Luminal B. Due to the high expression of proliferation-related genes, such as GGH (gamma glutamyl hydrolase), NSEP1 (nuclease sensitive element binding protein 1) and CCNE1 (cyclin E1) or genes associated with alternative growth factor pathways, such as FGFR1 (fibroblast growth factor receptor 1), HER1 and Src (sarcoma proto-oncogene), Luminal B is insensitive to endocrine therapy and responds better to adjuvant chemotherapy (*118*). HER2-positive and basal-like subtypes are high-risk tumors, and both of them have poorer outcomes. However, whereas the HER2-positive subtype of breast cancer can be treated by targeting HER2, basal-like tumors characterized by the absence of ER, PR and HER2 expression have worse prognosis (*121*). Claudin-low type that is described as a kind of basal-like breast cancer identified by low expression of claudins 3, 4 and 7 has very poor prognosis and a great degree of chemotherapy resistance (*122*). Although normal breast-like tumors lack expression of ER, PR and HER2, they are not considered to be basal-like subtype due to the absence of CK5 and EGFR expression, and they present with an intermediate prognosis between luminal and basal-like subtypes (*118*).

### 1.3.2 Triple negative breast cancer

Of all breast cancer tumors, about 10-20% are TNBC. Compared with non-TNBC, TNBC tumors are more aggressive and have a higher proliferation rate. Due to the lack of targeted therapies, chemotherapy is the systemic therapy currently available. Patients with TNBC experience often relapse more quickly and have worse prognosis compared to the other subtypes (*123*).

In order to further determine the biomarkers of TNBC subtypes, six different TNBC subtypes are identified based on gene expression profiles, including BL1 and BL2 (basal-like 1 and 2), IM (immunomodulatory), M (mesenchymal), MSL (mesenchymal stem-like) and LAR (luminal androgen receptor) subtypes (*124*, *125*). More recent advances overlapping these six TNBC subtypes with the classification of intrinsic breast cancer subtypes provide five new molecular subtypes of TNBC: BL-TNBC (basal-like TNBC), ML-TNBC (mesenchymal-like

TNBC), I-TNBC (immune-associated TNBC), LA-TNBC (luminal/apocrine TNBC) and HER2e-TNBC (HER2-enriched TNBC) (*126*) (Table 2).

**Table 2. Subtypes of TNBC**

| **Whole-genome gene expression profiling** | |
|---|---|
| **Subtype** | **High expression** |
| BL1 | Ki67, cell division and DNA damage response pathways |
| BL2 | Ki67, proliferation-associated, DNA repair genes and growth factor signaling |
| IM | Immune cell processes and immune signal transduction pathways |
| M | PI3K and Wnt pathways |
| MSL | PI3K, Wnt pathways and mesenchymal stem cell-associated genes |
| LAR | Steroid synthesis and androgen/oestrogen metabolism, such as AR |
| **Overlapping with intrinsic breast cancer subtypes** | |
| **Subtype** | **characteristic** |
| BL-TNBC | DNA-repair deficiency, growth factor pathway expression |
| ML-TNBC | EMT and cancer stem cell features |
| I-TNBC | Immune-associated |
| LA-TNBC | Androgen receptor overexpression |
| HER2e-TNBC | HER2-enriched |

With this, multiple potential therapeutic targets for TNBC have been identified, such as AR (androgen receptor), EGFR (epidermal growth factor receptors), Bcl2 family members, BRCA family members, adhesion molecules and p53 (*127*, *128*).

### 1.3.3 Estrogen signaling and breast cancer

ERs belong to the nuclear receptor superfamily of ligand-activated transcription factors and have two isoforms (ERα and ERβ). Both of these two isoforms consist of a DNA-binding domain, a ligand-binding domain, and two transcriptional activation function domains. They

share a 56% homology in the ligand-binding domain. However, partly due to a lack of highly efficient and selective anti-ERβ antibodies to quantify ERβ protein levels, the function of ERα is more clearly defined. ERα, as a hallmark of hormone-dependent tumor growth, is linked to prognosis and response to endocrine therapy (*129*). It has been reported that ERβ could be expressed in ER-positive breast cancer tumors and play a role as a tumor suppressor gene and may increase the sensitivity of ER-positive breast cancer tumors to tamoxifen (*130, 131*). In recent years, it has been reported that the variants of ERβ play different roles in TNBC (*128*).

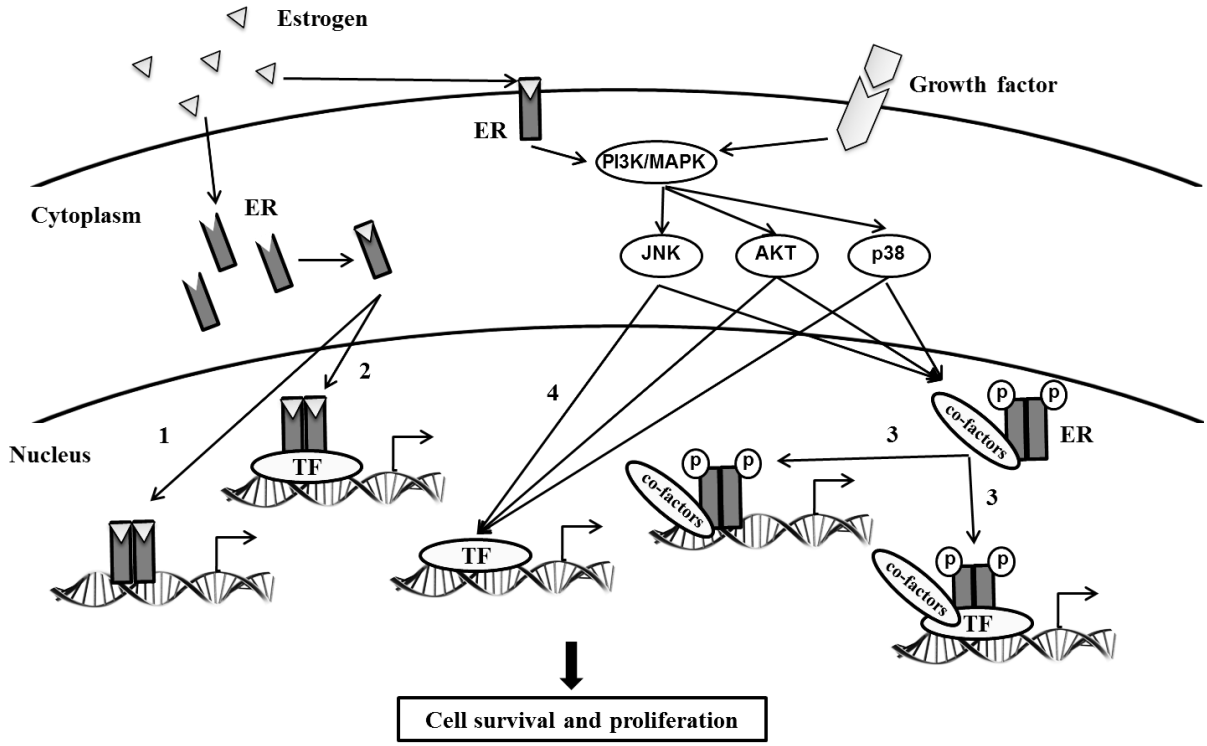


**Figure 6. ER signaling pathways**. Pathway 1. ER binds to specific estrogen response elements of target genes to activate gene expression. Pathway 2. ER regulates gene expression through interaction with other transcription factors. Pathway 3. ER and its co-factors modulate gene expression subsequent to phosphorylation via growth factor receptor signaling pathways. Pathway 4. This is a non-genomic pathway where membrane-localized ER regulates gene expression without binding to DNA directly.

Estrogen signaling can be broadly classified into genomic or non-genomic pathways (Figure 6). The classical genomic pathway of ER signaling infers that ER regulates gene expression through binding to specific estrogen response elements after that it has formed a dimer upon binding of E2 (17β-estradiol) (Pathway 1). ER also modulates gene expression by interacting with other transcription factors, such as AP-1, Sp1, NFκB, and RUNX1 (Pathway 2) (*129*).

Furthermore, ER can regulate target gene expression in a ligand-independent manner in which ER binds DNA directly or indirectly following ER activation through phosphorylation by protein kinases, including PI3K and MAPK kinases (Pathway 3). Finally, in the non-genomic pathway, membrane-localized ER can elicit rapid responses to E2 involving activation of the PI3K/MAPK signaling pathway, and thereby stimulate cell survival and proliferation without binding directly to DNA (Pathway 4) (*129, 132*).

### 1.3.4 AP-1 and breast cancer

Since AP-1 was identified as a transcription factor in 1987, the function of AP-1 has been broadly studied but not so much in breast cancer (Figure 7). It has been shown that AP-1, as a mediator of signal transduction, plays an important role in regulating cell proliferation, invasion and progression of breast cancer. AP-1 blockade in established breast tumors suppresses their growth in nude mice (*133*).

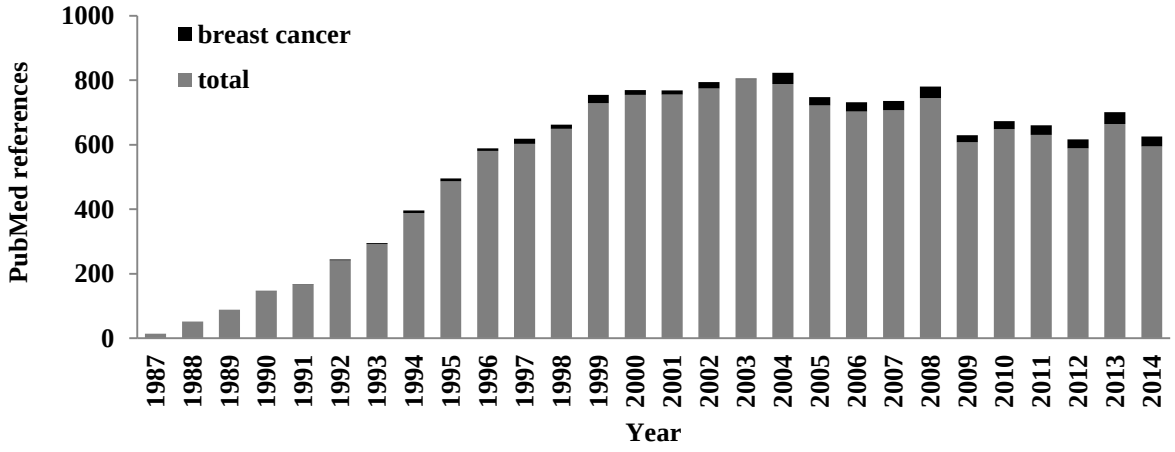


**Figure 7. Number of papers published between 1987 and 2014 according to a recent PubMed search using "AP-1 in breast cancer" and "AP-1".**

It is clear that AP-1 is involved in ER signaling pathways to modulate gene expression in ERα-positive breast cancer. Several studies have found that c-Fos is required for cell proliferation in breast tumors (*134*). Up-regulation of AP-1 is associated with tamoxifen resistance and increased invasiveness of MCF7 breast cancer cells and ERα-positive breast

tumors (*135-137*). Recently, it was reported that Fra1 expression is correlated with poor prognosis, corresponding to shorter overall survival and higher rate of lung metastasis, in ER-positive breast cancer patients (*138*). Furthermore, AP-1 family members, c-Fos and c-Jun, are involved in TGFβ1-mediated EMT in MCF7 cells (*139*).

There have not been many studies about the function of AP-1 in TNBC. It has been reported that compared to ER-positive breast cancer, Fra-1 is overexpressed and involved in cell proliferation, invasion and migration in ER-negative breast cancer cells (*140*). However, the mechanistic aspects of the modulatory effect of AP-1 in breast cancer, especially in TNBC are still unclear.

### 1.3.5 Therapeutic treatment of breast cancer

Surgery, radiation therapy, chemotherapy, endocrine therapy and targeted therapy are treatments of breast cancer (*141*). Radiation therapy is used to ensure that all the cancer cells are killed after surgery, and can shrink tumors in combination with chemotherapy. Endocrine therapy, such as the ER antagonist tamoxifen blocks the activity of ER in ER-positive breast cancer. An example of targeted therapy is trastuzumab that is a humanized anti-HER2 monoclonal antibody used for HER2-positive breast cancer tumors. Chemotherapy is used in the majority of ER-negative breast cancer patients, especially TNBC cases (*142*). However, chemotherapy can lead to hair loss, suppressed immune system and other serious negative side effects. Altogether, the above necessitates the identification of novel therapeutic strategies for TNBC patients.

## 1.4 Targeting transcription factors for cancer therapy

During tumorigenesis, some transcription factors are wrongly modulated by cellular pathways to overexpress or repress target genes and subsequently drive cancer cell biology. Thus, targeting transcription factors is a possible strategy for cancer therapy. Many transcription factors have been identified as potential therapeutic targets for cancer treatment, such as NFκB, STAT3, Notch, ATF5, HOX and AP-1 (*143*).

There are several ways to target transcription factors, including directly targeting the transcription factor; blocking protein-DNA or protein-protein interactions; destabilization of the mRNA of these transcription factors using siRNA (small interfering RNA) or antisense oligonucleotides; alteration of the functions and gene expression patterns of transcription factors using enzymatic regulators (*144*) (Table 3).

**Table 3. Examples of mechanisms to target transcription factors**

| Mechanism | Example | Transcription factor | Disease |
|---|---|---|---|
| Direct target | Tamoxifen | ER | Breast cancer |
| Protein-protein interactions | ABT-199 | BCL2 | Chronic lymphocytic leukaemia |
| Protein-DNA interactions | T-5224 | AP-1 | Arthritis |
| RNA degradation | AZD9150 | STAT3 | Lymphoma |
| Enzymatic regulator | Ruxolitinib | STAT proteins | Myoproliferative neoplasms |

However, unlike kinases that have fairly good drugable surfaces or cavities for binding of small molecules that alter their activity, transcription factors are more difficult to target due to the large surfaces for DNA and protein interactions. Additionally, their nuclear localization makes access of therapeutic agents more difficult.

## 1.5 Inflammation and cancer

In tissue microenvironments, leukocytes including neutrophils, monocytes and eosinophils will be activated and directly migrate from the venous system to sites of tissue injury to activate a multifactorial network of chemical signals and thereby cause inflammation for healing the injured area.

Neutrophils are the first recruited effectors after that the acute inflammatory response is activated. Macrophages are then activated and become the main source of cytokines which can cause acute inflammation that can develop into chronic inflammation (*145*). In the last decades, chronic inflammation has been identified as an inducer of various cancers (*146*).

Chronic inflammation induces alterations of both epithelial and stromal elements that in turn can lead to tumor initiation and tumor cell growth (Figure 8).

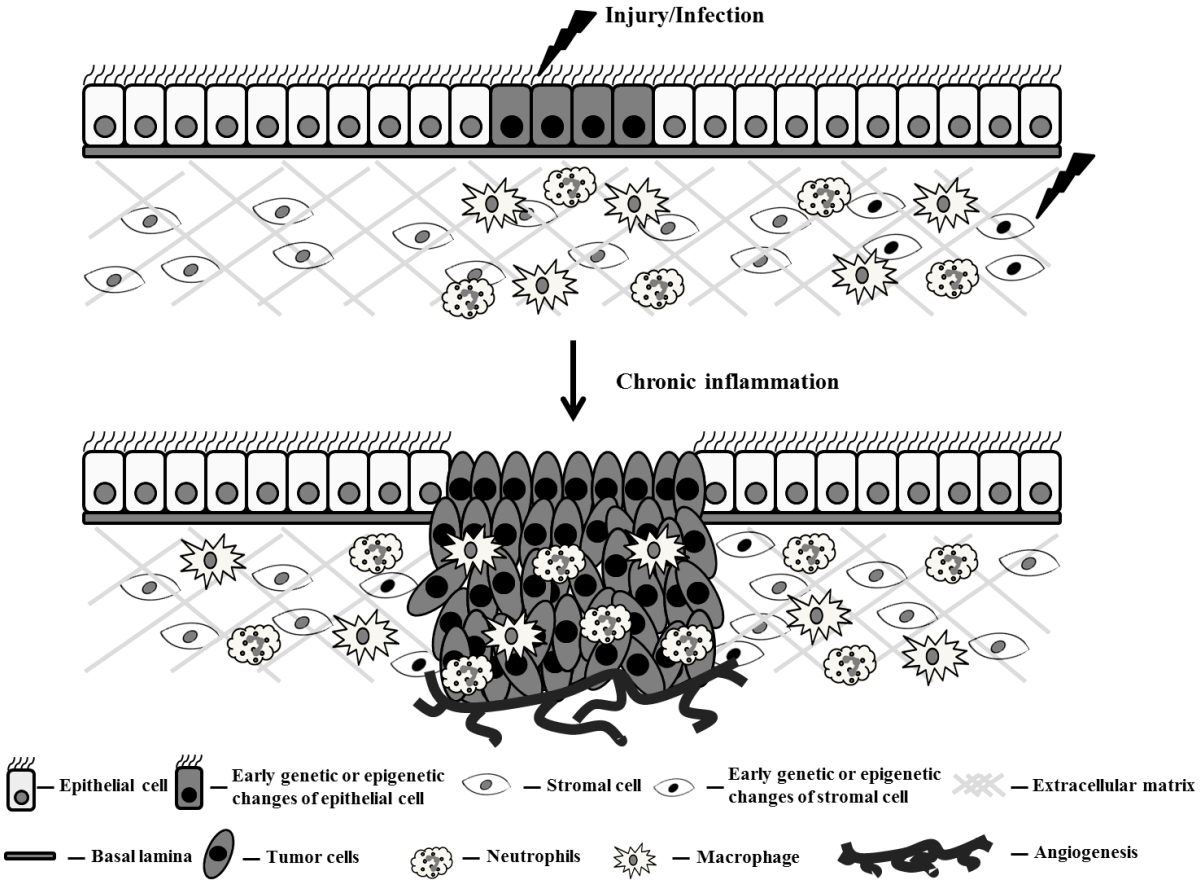


**Figure 8. Chronic inflammation microenvironment.** After tissue injury or infection, both epithelial and stromal cells are prone to mutagenesis and hyperplasia. Cytokines which are mainly released from macrophages may cause chronic inflammation, than further promote tumor cell hyperplasia and activation of neovascularization.

The relationship between inflammation and development of cancer is complex. Cytokines, such as TGF-β, play a role as tumor suppressors in early stages of tumor microenvironment (*147*). In contrast, exposure to pro-inflammatory cytokines, such as IL-6, IL-8 and TNFα (tumor necrosis factor α), transforms cells into a malignant career with changes resulting in cell proliferation and invasion (*148*).

### 1.5.1 TNFα

TNFα was discovered in 1975 (*149*). The human TNFα gene was cloned in 1985 (*150*). TNFα is an inflammatory cytokine that is mainly produced by macrophages, but also by a variety of other cell types such as NK cells, CD4+ lymphocytes, neutrophils, mast cells, and neurons (*145*).

In the tumor microenvironment, TNFα induces cell survival, EMT and further increases the expression of TNFα itself and other cytokines by the malignant cells (*151*). Interestingly, high-doses of TNFα, locally administered in breast cancer, show anti-angiogenic and anti-tumor effects (*152*). However, irrespective of whether the function of TNFα is pro-tumor or anti-tumor, there is no doubt that TNFα plays an important role in cancer.

### 1.5.2 TNFα signaling

TNFα exerts its biological functions by binding to its two receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNFR1 is expressed broadly in most cell types. TNFR1 can be fully activated by soluble and membrane-bound forms of TNFα. TNFR2 is only expressed in the immune system and is activated only by membrane-bound forms of TNFα. As opposed to TNFR1, TNFR2 plays mainly pro-survival functions as it does not have the death domain which TNFR1 has. However, if the cells have TNFR1 and TNFR2 co-expression, under certain conditions, such as under stress, TNFR2 may shift to TNFR1 signaling which can lead to apoptosis (*153, 154*).

As shown in Figure 9, after TNFα ligand binding to TNFR1 or TNFR2, TNFR-associated via the death domain (TRADD) can be recruited to TNFR1 or TNFR2, which in turn activates NFκB, p38-MAPK and JNK signaling pathways to regulate cell survival, proliferation and inflammation. In general, TNFα induces apoptosis through activating caspase cascades or JNK signaling pathways (*151*). However, when NFκB signaling is activated via IκB kinases ( IKK) complex which can be induced by both receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2) (*155*), the negative regulators of apoptosis such as cellular FLICE-like inhibitory protein (cFLAR), also known as FLIPL, BCL2 and superoxide dismutase will be induced that, in turn, interfere with apoptosis (*66, 153*).

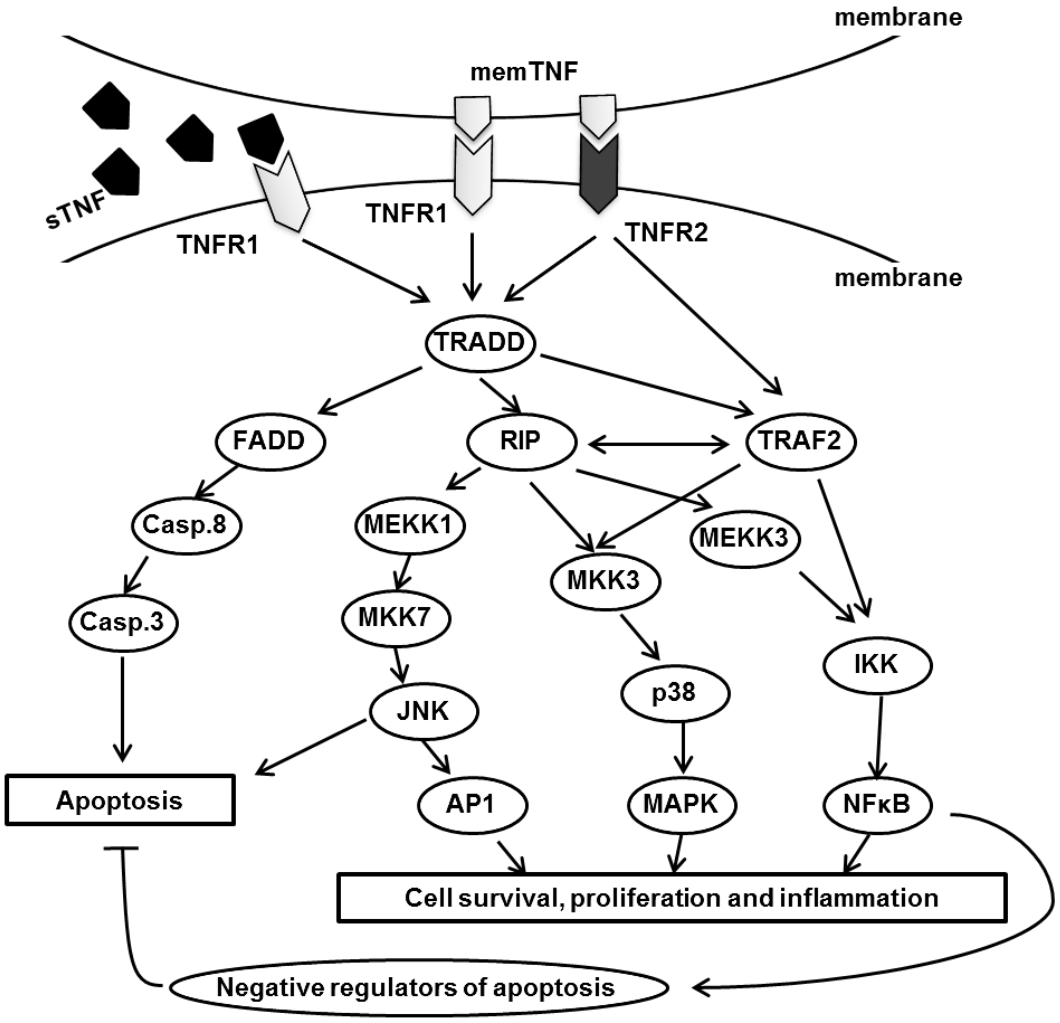


**Figure 9. TNFα signaling pathways.** TNFR1 and TNFR2 are activated by ligand-binding through memTNF or sTNF.TNFR1signalling triggers two different events. One is to stimulate caspase cascade signaling resulting in apoptosis. The other one is to induce JNK, p38-MAPK and NFκB signaling pathway that can result in cell survival, proliferation and inflammation. In addition, NFκB activation induces negative regulators of apoptosis that, in turn, inhibit apoptosis. Normally, TNFR2 stimulates pro-survival functions. When co-expressed with TNFR1 and under certain conditions such as under stress, TNFR2 can shift to TNFR1 signaling via recruitment of TRADD. memTNF, membrane-bound TNF; sTNF, soluble TNF; TRADD, TNFR-associated via death domain; FADD, FAS-associated via death domain; RIP, receptor interacting protein; TRAF2, TNF-receptor-associated factor.

## 1.6 Genome-wide studies of AP-1

Genome-wide study is to study transcription at a genomic scale, also referred to as transcriptomics. It relies on data from microarrays or high throughput sequencing (HTS) which allows rapid decoding of millions of DNA fragments at the same time. In addition,

HTS's low cost combined with high efficiency has led it to become a very popular method with applications in different areas of science.

Microarray technology has been widely used since the late 1990s (*156, 157*), and applied to cancer research since the 2000s. DNA microarray, one of the microarray technologies, has become an easy and rapid way to identify tumor-specific molecular markers for prognosis or potential therapy targets for cancer due to its relatively low cost (*158-160*).

ChIP (chromatin immunoprecipitation) technique was developed by Gilmour DS and Lis JT in 1980s (*161-164*). ChIP has become an important experimental technique to detect DNA-protein interactions and their specific genomic localization in the cells (Figure 10). ChIP-seq is ChIP assay combined with HTS. ChIP-seq can be used to map transcription factor binding sites and discover their networks (*165, 166*). Also, ChIP-seq can be used to discover distinct mechanisms underlying the transcription factor-mediated differential gene regulations (*167*). In addition, ChIP-seq is a useful technique to discover histone marks (*168*).

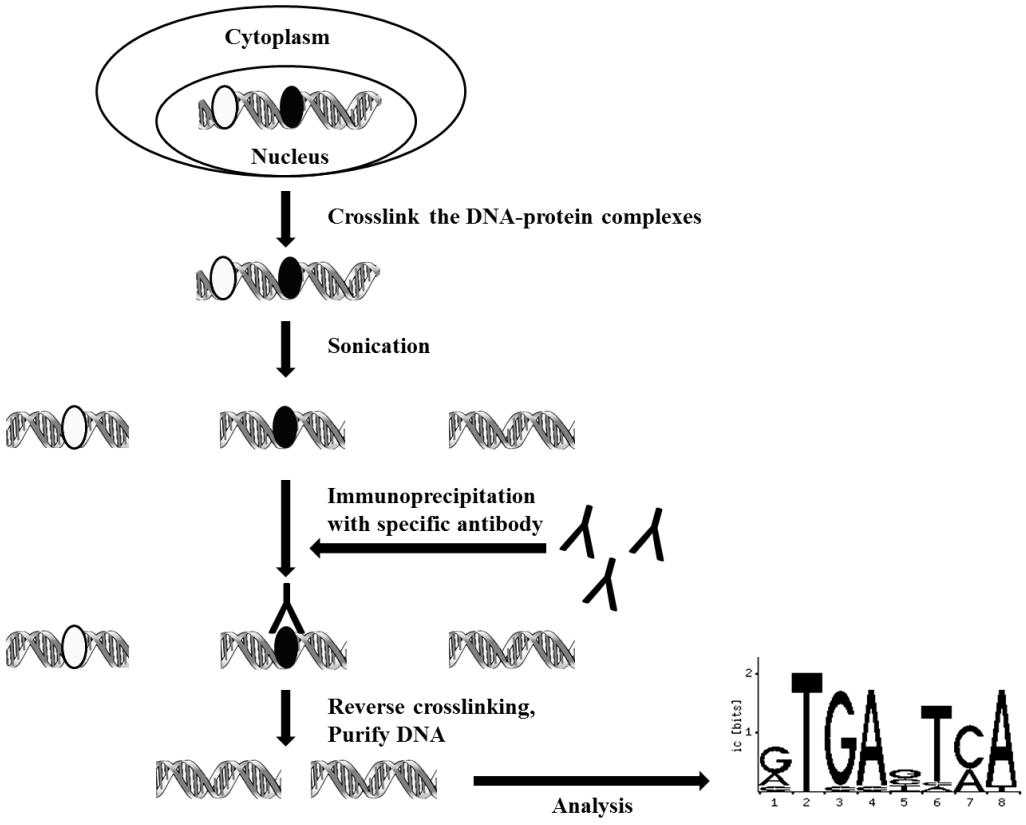


**Figure 10. Principle of ChIP assay.** DNA-protein complexes are crosslinked by formaldehyde. After that, the samples are sonicated into 200-1000bp fragments. By immunoprecipitation with specific antibodies, target protein-DNA complexes are pulled-down. DNA is ready to be analyzed after reverse crosslinking and further purification.

The development of microarray and ChIP-seq technologies provided researchers with high-throughput genomic tool to gain new insights into interactions between transcription factors and their regulatory networks. Using DNA microarrays, JunB and JunD were identified to be crucial for pathogenesis of primary cutaneous T-cell lymphomas (*169, 170*). By ChIP-seq analyses, Simon C et.al confirmed that AP-1 is critical for glucocorticoid receptor (GR) binding and its recruitment to co-occupied regulatory elements in a murine mammary epithelial cell line (*171*). In MDA-MB-231 breast cancer cells, Yes-associated protein/Tafazzin (YAP/TAZ), which does not carry a DNA-binding domain, need TEA domain family member (TEAD) factors co-occupying chromatin with AP-1 bound to composite regulatory elements to regulate gene transcription and to drive oncogenic growth (*172*). Combining ChIP-seq and microarray assay together, the direct target genes of JunD/c-Jun and JunD/ATF were identified in a rat model of crescentic glomerulonephritis (*173*).

Considering the important role of AP-1 in breast cancer as discussed above, a better and more highly understanding of molecular mechanisms of AP-1 signaling should provide important information that can potentially be explored to develop new therapies. Thus, exploring the genome-wide impact of transcription factor AP-1 in breast cancer is crucial.

# 2 AIMS OF THE THESIS

The overall aim of this project was to explore the genome-wide transcriptional regulatory networks of the transcription factor AP-1 in breast cancer cells with the ultimate hope that this knowledge will lead to novel strategies to develop candidate therapies for breast cancer patients in the future. In particular, the four specific aims were:

**I.** To address the role of AP-1 in gene expression programs including those controlled by estrogen-activated ERα.

**II.** To gain a genome-wide map of AP-1 binding and investigate mechanisms underlying AP-1-mediated gene regulation in TNBC cells.

**III.** To dissect the AP-1 – ZEB2 axis in TNFα-induced EMT in TNBC cells.

**IV.** To explore genome-wide analysis of AP-1 regulation of transcriptional programs in TNFα-stimulated TNBC cells.

# 3 METHODOLOGICAL CONSIDERATIONS

Significant considerations and limitations of the major methods used in this thesis are discussed below.

## 3.1 Cell lines

There are 16 different breast cancer cell lines used in this thesis. T47D, MCF7, MDAMB175, ZR751, SKBR3, HCC1569, MDAMB453, HCC202, HCC1954, MDAMB231, BT549, Hs578T, Sum159 and MDAMB157 cells were purchased from the American Type Culture Collection (ATCC). Whole-cell lysates of MDAMB361 and HCC70 which were used in paper II were obtained from Biomiga. The 16 different breast cancer cell lines can be classified into 3 groups: an ER-positive group, a HER2-positive group and a triple-negative group (no ER, PR and HER2 expression) based on gene expression profiles (*174, 175*).

Cell lines can be cultured infinitely, be easy to grow, are useful models and widely used for breast cancer research. However, results derived from the same subtypes are not exactly the same, even results from the same cell line studied in different labs can be different. That is because the origin of cells is different which leads to distinct signaling networks, although they are classified into the same subtypes based on gene expression. The culture conditions and passage numbers of the same cell line can also influence results in different labs (*176*).

In this thesis, we choose BT549 as an *in vitro* model for TNBC due to its high expression of AP-1 and that it is easier to transfect compared to other TNBC cell lines, such as MDAMB231 and Hs578T.

## 3.2 Quantitative polymerase chain reaction

Quantitative real time polymerase chain reaction (RT-PCR, qPCR) is widely used to measure gene expression. Two methods are common. In one, the SYBR Green dye which binds the minor groove of double-stranded DNA is used to quantitate the production of PCR products. Thus, if nonspecific products or primer-dimers are present in the SYBR Green dye assay, it will generate false positive signals. The other, TaqMan uses a fluorogenic single-stranded oligonucleotide probe which contains a fluorescent reporter dye at the 5’ end of the probe and a quencher dye at the 3’ end of the probe, to quantitate the production of PCR products. Only

specific PCR product can generate a fluorescent signal in TaqMan qPCR. Therefore, compared to SYBR Green dye based method, the TaqMan probe assay is more sensitive but generates lower calculated expression levels (*177*). However, there is no intrinsic reason that TaqMan probe assay would be required over SYBR Green dye assay. We have mainly used the SYBR Green dye assay in this thesis due to its lower cost. In addition, we have checked the PCR efficiency, confirmed that the SYBR Green dye assay amplified a single product using a denaturation curve and obtained the similar results as TaqMan probe assay.

### 3.3 Gene expression microarray analysis

High-throughput gene expression approaches such as microarrays and RNA-sequencing (RNA-seq) are able to measure expression of thousand genes in one sample and provide global out-look on complex biological processes at the same time. Based on massive parallel DNA sequencing technology, RNA-seq is a powerful method for research in discovering, profiling, and quantifying RNA transcripts. Despite the rapid advance in RNA-seq approaches, microarrays still remain the most popular and widely used techniques for whole genome expression profiles, especially in humans.

In this thesis, we used two technologies, Agilent SurePrint G3 Human GE 8x60K array which is ink-jet technology and Affymetrix human Gene 1.1 ST array which is photolithography technology, to study global gene expression. Agilent arrays have 60mers oligonucleotides which are longer than those used in the Affymetrix arrays (25mers), but lower density arrays than Affymetrix. The Affymetrix platform is the earliest technology used in studies of global gene expression and has been used since 1989 and it remains widely used. Compared to other gene expression arrays, Affymetrix has a rich bioinformatics architecture that allows for a broad range of analyses that provide more clues for the further study.

A higher cutoff for fold change will decrease the sensitivity but increase the specificity. In contrast, a lower cutoff fold will increase the sensitivity but decrease the specificity. In order to have good sensitivity and specificity of the analysss, we applied a cutoff fold of 2.0 or 1.5 and *p*-value less than 0.05 for significantly modulated gene expression.

### 3.4 Chromatin immunoprecipitation (ChIP)

ChIP is a powerful tool and a direct way to identify DNA-protein interactions and their specific genomic localization in living cells. Good and clear ChIP results are dependent on cell numbers, the range of chromatin fragments after sonication and the specificity of the antibody in this particular. However, tissue and cell specific effects also influence the results. Data might not be the same for different cell lines, even though they belong to the same subtype. Due to the dynamics of cells, samples collected at different time points may show various intensities of signals.

### 3.5 Small interfering RNA (siRNA)

siRNA refers to the synthetic generation of RNA interference. siRNA can be easily and rapidly transfected into cells to reduce the expression of a specific gene at the mRNA level. Unfortunately, siRNAs might exert unspecific binding to similar sequences to those of the target gene to cause so called off-target effects. Thus, it is better to use at least two different siRNAs to target one gene. Another challenge for this technique is innate immunity which may influence the knockdown efficiency in different cell types, for example, the knockdown efficiency of AP-1 in MDAMB231 cells poorer than BT549 cells.

### 3.6 Cell proliferation assays

In this thesis, we use two methods, BrdU (Bromodeoxyuridine-labeling) and WST-1 (2 - (4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2Htetrazolium, mono-sodium salt), to measure cell proliferation. BrdU is a common method to measure cell proliferation where the thymidine analog reagent, BrdU, incorporates into DNA during the S-phase of the cell cycle. BrdU has a high labeling efficiency and can be directly detected under the microscope. WST-1 is a method to measure cell viability through the amount of a soluble formazan salt that is related to the metabolic activity of living cells that can be determined in an ELISA plate reader. Data from WST-1 is dependent on the number of living cells and the incubation time after the WST-1 solution added into the medium.

### 3.7 Cell invasiveness

To study cell invasion ability, we choose to use BD BioCoat$^{TM}$ Matrigel Invasion Chambers which is a low throughput and highly efficient quantitative method. The results are dependent on the cell types because different cell types invade at different rates, and are also dependent on specific conditions, such as incubation time, cell seeding density and chemoattractant. In this thesis, we found that TNBC cells in the upper chamber with 0% FBS medium migrated to the lower chamber containing 10% FBS medium and the number of cells in the lower chamber was determined after 24 hours.

### 3.8 Apoptosis assay

In this thesis, we use two methods to detect apoptosis. One is to measure the expression of cleaved caspase 3, which is a marker of apoptosis, at the protein level using Western blot analysis. Another method is the Cell Death Detection ELISA$^{PLUS}$ kit from Roche that measures the amount of nucleosomes from living cells. Compared to Western blot based cleaved caspase 3 assay, the Cell Death Detection ELISA$^{PLUS}$ kit is more sensitive. β-actin is used as internal control to quantitate the expression of cleaved caspase 3, whereas for the Cell Death Detection ELISA$^{PLUS}$ kit, it is important that the number of cells in different samples is equal before measuring the apoptosis, as the kit is a colorimetric assay.

# 4 RESULTS AND DISSCUSSION

## 4.1 PAPER I: Interplay between AP-1 and estrogen receptor α in regulating gene expression and proliferation networks in breast cancer cells

ERα mediates a proliferative effect in ER-positive breast cancer. Studies of mapping ER-binding regions show that AP-1 motifs are enriched within the regions bound by ERα in breast cancer cells (*178, 179*). AP-1 has been reported to play a critical role in regulating breast cancer cell proliferation (*134, 140*). However, the interplay between ERα and AP-1 signaling pathways at the chromatin level is still unclear. Thus, in paper I we addressed the role of AP-1 in the gene expression programs including those controlled by estrogen-activated ERα.

We used MCF-7 cells and silenced c-Fos expression through RNA interference system. Changes in gene expression were studied using microarray and real-time PCR. Our analysis identified 37% of all estrogen-regulated genes were attenuated by silencing of c-Fos. In addition, among the genes which were regulated by ERα, 25% of ERα-induced genes and 47% of ERα-repressed genes were affected by c-Fos knockdown. These results suggest that c-Fos is a major contributor to ERα-mediated gene regulation.

Gene ontology analysis of all ERα regulated genes that were affected by c-Fos knockdown showed that the most overrepresented category for E2-induced genes was related to the G1-S transition of the mitotic cell cycle, and for E2-repressed genes related to negative regulation of cell proliferation. We evaluated enrichment of specific gene functions for the groups of genes whose estrogen regulation was affected by c-Fos knockdown and found proliferation highest ranked. These findings implicate a critical role of c-Fos in the regulation of cell proliferation and the mitogenic effect of E2 in ERα-positive breast cancer cells.

In breast cancer cells, it has been reported that ERα promotes cell proliferation by regulating the expression of E2F1 that is well known for its involvement in cell proliferation, differentiation and apoptosis (*180, 181*). In this study, pathway analysis revealed that silencing of c-Fos downregulated an E2F1-dependent pro-proliferative gene network. Additionally, ChIP-qPCR assays showed for the first time that c-Fos and ERα can cooperate in regulating E2F1 gene expression by binding to regulatory elements in the E2F1 promoter.

To study the molecular details of the cross talk between AP-1 and estrogen signaling, we identified PKIB (cAMP-dependent protein kinase inhibitor-β) as a novel ERα/AP-1 target molecule. PKIB has been reported to promote cell proliferation in prostate cancer (*182*). WST-1 assay and bromodeoxyuridine (BrdU)-labeling experiments showed that PKIB silencing inhibits cell proliferation in ER-positive breast cancer cells. In addition, using the Oncomine database we found that the expression of PKIB is higher in ERα-positive compared to ERα-negative breast cancer tissues.

In conclusion, we demonstrated that AP-1 is a major contributor to ERα-mediated gene regulation. We identified PKIB as a novel ERα/AP-1 target molecule that is important for growth of breast cancer cells.

## 4.2 PAPER II: Genome-wide profiling of AP-1-regulated transcription provides insights into the invasiveness of triple-negative breast cancer

AP-1 plays a key role in a variety of cellular processes. In paper I was shown that AP-1 is a major contributor to ERα-mediated gene regulation and promotes cell proliferation. It has been reported that compared to ER-positive breast cancer, one of the AP-1 family members, Fra-1, is overexpressed and involved in cell proliferation, invasion and migration in ER-negative breast cancer cells (*140*). However, mechanism of action of AP-1 in TNBC remains largely unknown. In this study, we aimed to gain a genome-wide map of AP-1 binding and investigate mechanisms underlying AP-1-mediated gene regulation in TNBC cells.

First, using publicly available gene expression profiling datasets we observed that among all of the AP-1 family members, Fra-1 is overexpressed in basal-like breast cancer which is described as very similar to TNBC (*183*), and associated with poor prognosis. Data from Western blot and qPCR showed that both c-Jun and Fra-1 were highly expressed in TNBC cell lines.

We choose as our major cell model one of the analyzed TNBC cell lines, BT549 which represents basal-like subtype breast cancer based on global gene expression analysis (*184*). In addition, we found that this cell line had a high transfection efficiency of siRNA compared to other TNBC cell lines (Hs578T and MDAMB231). Based on this cell model, we identified 11670 Fra-1 binding regions and 14201 c-Jun binding regions, and that most of them overlap. This suggested that they form heterodimers. In addition, the majority of these binding regions

were distal to the promoter in intergenic and intronic regions. As expected, *de novo* DNA motif search revealed that the AP-1 motif was the most enriched motif, 58% of Fra-1 binding regions and 47% of c-Jun binding regions.

Global gene expression microarray analysis for TNBC cells upon Fra-1 or c-Jun knockdown was used to gain further insight into AP-1-regulated transcriptional networks. Our analysis identified differential expression of 419 and 690 genes upon depletion of Fra-1 and c-Jun, respectively. Among these genes, 222 genes regulated by both Fra-1 and c-Jun were associated with cytokine-mediated signaling, type I interferon-mediated signaling, chemotaxis, cell adhesion, immune response, cell junction assembly, adherens junction organization and inflammatory response.

Moreover, we found that the proliferative phenotypes of TNBC cells were inhibited upon depletion of both Fra-1 and c-Jun. Cluster analysis of Fra-1 and c-Jun common cell proliferation target genes revealed that 20 proliferation-repressing genes were up-regulated upon Fra-1 and c-Jun knockdown, whereas 7 proliferation-promoting genes were down-regulated. Among these genes, the CLCA2 (chloride channel accessory 2) gene was the most up-regulated transcript upon Fra-1 and c-Jun knockdown and shown to play an important role in inhibition of cancer cell migration and invasion (*185, 186*). We further found that Fra-1 and c-Jun repress CLCA2 expression through direct binding to its intron region to promote cell proliferation.

In addition, silencing of Fra-1 and c-Jun reduced the invasion ability both *in vitro* and *in vivo*. It is known reported that ZEB2 represses the transcription of E-cadherin that is one of the hallmarks of EMT resulting in metastasis and invasion (*187, 188*). In this study, we showed that ZEB2 was one of AP-1 direct target genes and was overexpressed in TNBC cells.

In an effort to elucidate the mechanism behind AP-1 transcriptional activity in TNBC cells, two signaling pathways, PI3K/Akt and MAPK/ERK that are known to be activated and are associated with a poor prognosis in TNBC (*189, 190*), were studied by using two pharmacologic inhibitors. Our results showed that high PI3K/Akt or MAPK/ERK activity positively regulated Fra-1 and c-Jun expression and was associated with increased AP-1 transcriptional activity in TNBC cells.

Taken together, we found that AP-1 is overexpressed in TNBC and associated with poor prognosis. AP1 repressed CLCA2 expression through direct binding to its intron region to

promote cell proliferation. Furthermore, we confirmed that AP-1 mediates down-regulation of E-cadherin through direct transcriptional induction of ZEB2 (Figure 11).

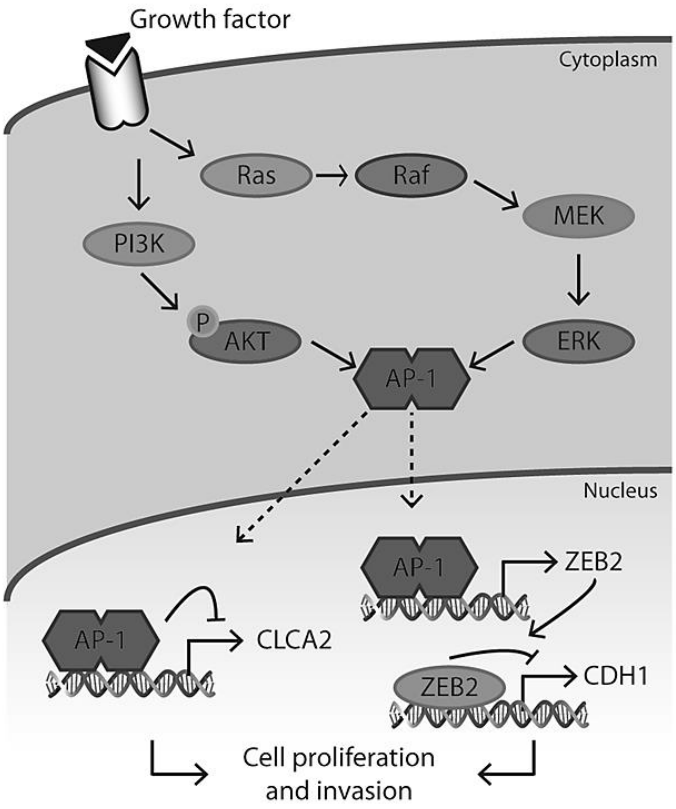


**Figure 11. Proposed model of AP-1 promoting cell proliferation and invasion in TNBC.**

## 4.3 PAPER III: AP-1-mediated chromatin looping regulates ZEB2 transcription: new insights into TNFα-induced epithelial-mesenchymal transition in triple-negative breast cancer

TNFα is an inflammatory cytokines is highly expressed in breast tumors as compared with normal tissues and involved in EMT in breast cancer (*191*). To expand our finding in paper II we decided to dissect the AP-1 – ZEB2 axis in TNFα-induced EMT in TNBC cells.

Two different TNBC cell lines, BT549 and Hs578T, were used as cell models in this study. First, we found that stimulation of cells with TNFα resulted in morphological changes of TNBC cells and the expression of EMT markers, confirming that TNFα induces EMT in our model TNBC cells. Furthermore, we confirmed our hypothesis that TNFα-mediated EMT in

TNBC cells is dependent on AP-1 – ZEB2 signaling through PI3K/Akt and MAPK/ERK pathways.

Based on published spliced transcripts, two alternatively spliced 5'UTR isoforms of the ZEB2 gene, referred to as exon 1a (from ENST00000558170) and exon 1b (from ENST00000409487), were found to be expressed in breast tumor samples and highly expressed in TNBC cell lines which is in agreement with our previous findings that ZEB2 was overexpressed in TNBC cell lines.

Based on our AP-1 ChIP-seq data, ZEB2 gene harbors only one AP-1 binding site that is located in the promoter 1b region. However, both of these two transcripts were regulated by AP-1 with or without TNFα treatment, which indicates that AP-1 binding to promoter 1b also regulates the activity of promoter 1a by long range chromosomal interactions. We performed the chromosome conformation capture (3C) assay to confirm our hypothesis. Our results showed that AP-1, when activated by TNFα, binds to a site in promoter 1b of the ZEB2 gene where it regulates the expression of both promoter 1b and 1a, the latter via mediating long range chromatin interactions (Figure 12).

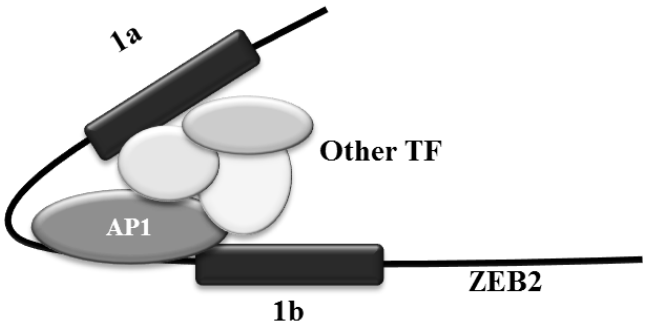


**Figure 12. Proposed model of AP-1-mediated chromatin looping regulating ZEB2 transcription.**

In summary, our study not only reveals a critical mechanism underlying inflammation-induced metastatic potential in TNBC but also suggests AP-1, its upstream regulatory signaling pathways ERK and Akt and ZEB2 as potential drug targets for reversing EMT, the key event in the acquisition of invasive potential.

## 4.4 PAPER IV: AP-1 is a key regulator of TNFα-mediated triple-negative breast cancer progression

TNFα as a pro-inflammatory cytokine can facilitate tumor progression and metastasis. However, the molecular mechanisms of TNFα-mediated TNBC progression are not well described. From our previous studies, we found that AP-1 proteins, mainly Fra1 and c-Jun, are overexpressed in TNBC compared to the other subtypes of breast cancer. Also, TNFα triggered EMT through up-regulation of ZEB2 via AP-1 activation in TNBC. Although it has long been known that AP-1 plays a key role in response to inflammatory signaling, the molecular mechanisms are still unclear. In paper IV was explored the AP-1 cistrome and transcriptome in response to TNFα, this to better understand the mechanism underlying inflammation-induced tumor progression.

In this study, we used BT549 cells as our cell model, and Hs578T as another TNBC cell line to confirm our results. To begin to explore the AP-1 cistrome and transcriptome in response to TNFα, we performed ChIP-seq to map the c-Jun binding regions in BT549 cells upon TNFα treatment for 3 hours. First, we identified 13800 and 4570 c-Jun binding regions in TNFα stimulated and non-stimulated cells, respectively. As expected, *de novo* DNA motif search revealed that AP-1 motif was the most enriched motif in c-Jun cistromes in both TNFα treated and non-treated groups. The major genomic positions of c-Jun binding regions were found in intergenic and intronic parts of genes. Through use of the genomics regions enrichment of annotation tool (GREAT), we found that c-Jun binding regulates apoptosis signaling pathways and oxidative stress responses upon TNFα stimulation.

To identify global gene expression regulated by c-Jun upon TNFα stimulation, we used microarray and real-time PCR. BT549 cells were transfected by siRNA to knockdown the expression of c-Jun with or without TNFα treatment. We identified that the expression of 1192 genes and 940 genes in control cells and c-Jun-depleted cells, respectively, were differentially regulated by TNFα treatment. By comparing the si_control to si_c-Jun group in the presence of TNFα, we identified 616 genes that were affected by decreased c-Jun expression. Overlapping these 616 genes with TNFα-induced c-Jun binding regions within 20kb upstream or downstream of a known transcriptional start site (TSS), we identified 204 direct c-Jun target genes in TNFα-stimulated cells, such as EGR2, ZEB2, MMP9 and TNFAIP8 which were also confirmed by qPCR. Gene ontology analysis of these 204 genes showed that significantly overrepresented categories were related to cell proliferation,

phosphorylation, cell adhesion, cell motion, intracellular signaling cascade, gene expression, apoptosis and cellular homeostasis, but not related to the inflammatory response.

To further study the functions of c-Jun in response to TNFα, knockdown of c-Jun was done and cells were treated with or without TNFα. We found that c-Jun knockdown sensitized TNBC cells to TNFα-induced apoptosis, and inhibits TNFα-induced cell invasion. Next, we wanted to identify c-Jun direct target genes with known functions in apoptosis and cell invasion. Twenty-three anti-apoptotic genes such as Ninj1, 13 pro-apoptotic genes such as CDK5, 14 pro-invasion genes such as ZEB2 and 5 anti-invasion genes such as CLCA2 were identified. Furthermore, high expressions of these pro-invasion genes were associated with poor outcome in breast cancer, especially in basal tumors. To further describe the mechanisms of TNFα-regulated cancer progression via c-Jun activation, we focused on the c-Jun direct target gene Ninj1. It has been shown that Ninj1 deficiency suppresses cell proliferation but enhances apoptosis and premature senescence in a p53-dependent manner in colon cancer cells (*192*). In line with this, we found that Ninj1 knockdown enhanced apoptosis and reduced the invasiveness of TNBC cells upon TNFα stimulation. Furthermore, the effect of c-Jun knockdown on apoptosis and cell invasion was reversed by overexpression of Ninj1. Together, these results demonstrate that the Ninj1 gene, which is transcriptionally regulated by c-Jun, drives TNFα-induced malignant characteristics of TNBC cells.

TNFα is well known as a central regulator of inflammation. In our study, we identified a region of TNFα-induced c-Jun binding sites flanking the CXC chemokine cluster (IL8, CXCL1, CXCL2 and CXCL3) on chromosome 4. However, we found that c-Jun is not essential for expression of these chemokine genes. It is well known that NFκB is a master effector of TNFα-induced inflammation (*193, 194*). The above described CXC chemokine cluster region was earlier found to harbor NFκB binding sites induced by IL1 (*195*). Based on *de novo* DNA motif analysis, we found that the NFκB motif was enriched in the presence of TNFα. In addition, TNFα induced recruitment of NFκB to the CXC chemokine gene cluster, and knockdown of NFκB dramatically reduced the expression of these chemokine genes upon TNFα stimulation. Accordingly, gene ontology analysis did not find c-Jun direct target genes associated with inflammatory response in the presence of TNFα, suggesting that NFκB activation was essential for induction of the chemokine gene expression in response to TNFα. ChIP-qPCR was used to further investigate the relationship between NFκB and c-Jun recruitment to the CXC chemokine cluster. Knockdown of NFκB reduced the recruitment of c-Jun to the promoters of chemokine genes. In contrast, knockdown of c-Jun increased the

recruitment of NFκB. These findings indicate that NFκB might be a pioneer factor for c-Jun recruitment to this gene cluster (Figure 13).

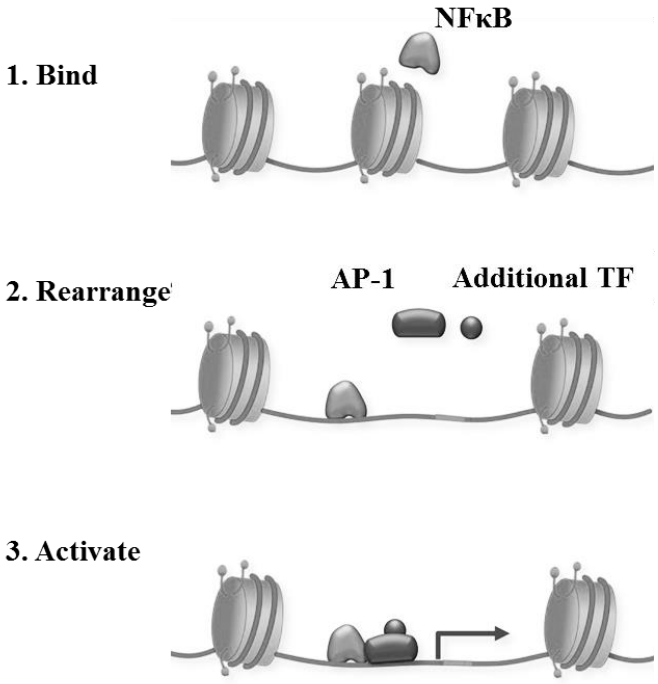


**Figure 13. NFκB as a pioneer factor for AP-1 recruitment to the chemokine gene cluster.** The function of NFκB is to induce and maintain an open chromatin architecture, then AP-1 and other transcription factors can bind to DNA to regulate gene expression.

In conclusion, we describe for the first time the inflammatory cistrome for the AP-1 transcription factor c-Jun in TNBC cells. Furthermore, we demonstrate that AP-1 activation in TNBC cells contributes to inflammation-induced tumor progression, rather than the inflammatory response. We identify a set of c-Jun-regulated pro-invasion genes that are strongly associated with clinical outcomes in TNBCs. We characterize the Ninj1 gene, which is transcriptionally regulated by c-Jun to drive TNFα-mediated malignant characteristics of TNBC cells.

# 5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

## 5.1 AP-1 in ER-positive breast cancer

AP-1 plays important roles in carcinogenesis and cancer progression. It is clear that AP-1 is involved in ER signaling pathways to modulate gene expression in ERα-positive breast cancer. In this thesis, we found that one of the AP-1 family members, c-Fos, is a major contributor to ERα-mediated gene regulation. Moreover, we identified a novel ERα/AP-1 target molecule, PKIB, which is important in breast cancer growth. In addition, evidence is provided that c-Fos is involved in E2-induced activation of E2F1 expression through binding to the E2F1 promoter to regulate cell cycle progression.

Thus, a detailed understanding of ERα/AP-1/E2F1 network in relation to proliferation of breast cancer cells will facilitate identification of components of this network suitable as drug targets in order to inhibit breast cancer cell proliferation. In addition, it has earlier been reported that up-regulation of AP-1 is associated with tamoxifen resistance in MCF7 breast cancer cells and ERα-positive breast tumors (*135-137*). A more clear understanding of the mechanism of AP-1 signaling may suggest novel strategies to overcome tamoxifen resistance.

## 5.2 AP-1 in TNBC

Compared to other subtypes of breast cancer, TNBC has yet no specific target and is only sensitive to chemotherapy and radiation therapy. Patients with TNBC experience often relapse more quickly and have worse prognosis compared to other breast cancer subtypes (*123*).

The most important finding in this thesis is that AP-1 is overexpressed in TNBC and associated with poor prognosis. Also that AP-1 represses CLCA2 expression through direct binding to its intron region to promote cell proliferation and that AP-1 mediates downregulation of the important EMT regulator, E-cadherin, through direct transcriptional induction of its repressor ZEB2.

TNFα, one of the inflammatory cytokines, promotes tumor development and progression (*191*). In this thesis, is found that TNFα induces EMT in TNBC cells via activation of AP-1 signaling and expression of the EMT regulator ZEB2. Furthermore, we defined for the first

time the inflammatory cistrome of the AP-1 transcription factor c-Jun and demonstrated that AP-1 activation in TNBC cells contributes to inflammation-induced tumor progression, rather than inflammatory response. A set of c-Jun-regulated pro-invasion genes was identified that are strongly associated with clinical outcomes in TNBCs. Described is also TNFα-induced c-Jun activation of Ninj1 gene expression driving TNBC cell tumor progression.

Together, these results suggest that AP-1 is an important and a valid therapeutic target for breast cancer. There are several AP-1 inhibitors that are currently under intense development pre-clinically and clinically in many therapeutic areas. One of the inhibitors of AP-1, T-5224, has advanced into human phase II clinical trials for rheumatoid arthritis (*196*). Future studies should focus on to identify if AP-1 inhibitors could be used in treatment of breast cancer.

# 6 ACKNOWLEDGEMENTS

This work was performed at the Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden. I would like to express my deepest appreciation to all my friends and colleagues who have helped me during these four years, thus making this thesis possible. I would like to especially thank:

**Chunyan Zhao**, my supervisor, I feel so lucky and so proud to be your first PhD student. Thank you for teaching me the scientific techniques and guiding me to become an independent scientist. You are like a babysitter and sister during my PhD, helping me with all kinds of difficulties. I enjoyed very much working with you and discussing all my scientific challenges with you. You are elegant, smart and without your scientific input and continuous advice, this dissertation would not have been possible. You are not only my supervisor, but also my best friend. I will also miss the time we spent going to gym, shopping and taste different delicious foods after work.

**Karin Dahlman-Wright**, my co-supervisor, no words could express my gratitude for giving me the opportunity to do a PhD in your group. I appreciate the excellent working environment that you have provided for this group. You are a great leader. You are always very busy but you never neglect to help and encourage me and maintain my confidence.

**Yawen Liu**, my master supervisor and our former colleague, you opened the first window for my scientific views and introduced me to the field of research conducted in Karin's group. You are an excellent supervisor, a great leader. Even though you are very busy now, but you never neglect to provide me with advice, throughout my PhD, my life in Sweden and my future career. I am grateful for all your support and guidance.

All the members in Karin's group for your support, friendship and for all the wonderful memories. **Lars-Arne Haldosén**, for your scientific advices, reading my manuscripts and thesis and for sharing your knowledge. **Min Jia** and **Hui Gao**, for nice company in the office and during lunch times. **Lucia Bialesová and Amirhossein Kharman Biz**, for nice company in the lab and best wishes to your research career. **Indranil Sinha**, for nice company in the office and for quickly analyzing our data. **Isabella Hübscher**, for your warm personality and nice conversations, and best wishes with your further study. **Huan He**, for being a new PhD student, a new "stacy" in this group and best wishes with your PhD study. I am sure that you will enjoy your time in this group and miss here just like I do after four years.

All my Chinese friends in Karolinska Institutet for providing a feeling of home around me. **Yumei Diao**, you are my first friend in Stockholm. I really enjoyed every moment we had and best wishes to you. **Jia Guo**, for your warm personality and good company in the weekends. Good luck with the rest of your PhD study and your life. **Wenbo Dong**, for sharing the room and taking care of me during my pregnancy. Best wishes to your happy married life. **Miao Zhao**, for lending me your maternity dress and providing me with advice during pregnancy. **Ting Zhuang**, for the fun company in the lab and after the work. You are really a nice sister. And all the boys in the Department of Biosciences and Nutrition, **Jiyu Guan**, **Rongrong Fan**, **Ning Liang**, **Zhiqiang Huang** and **You Xu**, for helping me move several times and the nice company in the lab. Especially, You Xu, thank you for helping me to find a stable living place.

The department's administration, for your warm personality and endless help. **Monica Ahlberg**, for helping me with all the tedious documents to make my PhD happened from my PhD registration, yearly follow-up, half-time control to my dissertation. **Marie Franzén**, for helping me to arrange my trip to Sweden, scheduling our group meetings and taking care all the documents which needed to be signed by Karin. Former member of administration, **Lena Magnell**, for arranging my first accommodation when I came to Stockholm.

**To all other members of BIONUTS**: none mentioned, none forgotten.

To all my best friends in China. **Dongdong** and **Shanshan**, who have been my friends more than 20 years. Thanks for a long lasting friendship, for taking me to wherever I needed to go and for always believing in me. **Weilin**, **Yuan** and **Qin**, for all fun times we had in China, for being great friends and listeners and for always supporting me in everything I do.

To my uncle and his **family** for helping me to improve my English.

To my families. **My Dear Tengyu**, thank you for all your love, understanding, encouragement and supports in all my adventures. **My son**, you are the best gift. Your birth brings so much love and happiness into my life. Most importantly, thanks to **my parents** and **my parents-in-law** for taking such a great care of my son during my PhD.

I love all of you!

# 7 REFERENCES


1. P. K. Vogt, Fortuitous convergences: the beginnings of JUN. *Nature reviews. Cancer* **2**, 465 (Jun, 2002).
2. W. Lee, A. Haslinger, M. Karin, R. Tjian, Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* **325**, 368 (01/22/print, 1987).
3. P. Angel, M. Karin, The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1072**, 129 (12/10/, 1991).
4. L. J. Ransone, J. Visvader, W. W. Lamph, P. Sassone-Corsi, I. M. Verma, fos and jun interaction: the role of the leucine zipper. *International journal of cancer. Supplement = Journal international du cancer. Supplement* **4**, 10 (1989).
5. L. J. Ransone, J. Visvader, P. Sassone-Corsi, I. M. Verma, Fos-Jun interaction: mutational analysis of the leucine zipper domain of both proteins. *Genes & development* **3**, 770 (Jun, 1989).
6. T. Deng, M. Karin, JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes & development* **7**, 479 (Mar, 1993).
7. P. Lopez-Bergami, E. Lau, Z. Ronai, Emerging roles of ATF2 and the dynamic AP1 network in cancer. *Nature reviews. Cancer* **10**, 65 (Jan, 2010).
8. T. D. Halazonetis, K. Georgopoulos, M. E. Greenberg, P. Leder, c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* **55**, 917 (12/2/, 1988).
9. T. Kouzarides, E. Ziff, The role of the leucine zipper in the fos-jun interaction. *Nature* **336**, 646 (Dec 15, 1988).
10. T. Hai, T. Curran, Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 3720 (May 1, 1991).
11. J. L. Sonnenberg, P. F. Macgregor-Leon, T. Curran, J. I. Morgan, Dynamic alterations occur in the levels and composition of transcription factor AP-1 complexes after seizure. *Neuron* **3**, 359 (Sep, 1989).

12. K. Kovary, R. Bravo, Existence of different Fos/Jun complexes during the G0-to-G1 transition and during exponential growth in mouse fibroblasts: differential role of Fos proteins. *Molecular and cellular biology* **12**, 5015 (Nov, 1992).
13. L. R. McCabe, M. Kockx, J. Lian, J. Stein, G. Stein, Selective expression of fos- and jun-related genes during osteoblast proliferation and differentiation. *Experimental cell research* **218**, 255 (May, 1995).
14. Y. Chinenov, T. K. Kerppola, Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* **20**, 2438 (Apr 30, 2001).
15. R. Venugopal, A. K. Jaiswal, Nrf2 and Nrf1 in association with Jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene* **17**, 3145 (Dec 17, 1998).
16. L. Chen, J. N. Glover, P. G. Hogan, A. Rao, S. C. Harrison, Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. *Nature* **392**, 42 (Mar 5, 1998).
17. G. Brodin, A. Ahgren, P. ten Dijke, C. H. Heldin, R. Heuchel, Efficient TGF-beta induction of the Smad7 gene requires cooperation between AP-1, Sp1, and Smad proteins on the mouse Smad7 promoter. *The Journal of biological chemistry* **275**, 29023 (Sep 15, 2000).
18. H. Wang, M. Birkenbach, J. Hart, Expression of Jun family members in human colorectal adenocarcinoma. *Carcinogenesis* **21**, 1313 (Jul, 2000).
19. J. Rangatia *et al.*, Elevated c-Jun expression in acute myeloid leukemias inhibits C/EBPalpha DNA binding via leucine zipper domain interaction. *Oncogene* **22**, 4760 (Jul 24, 2003).
20. S. Langer *et al.*, Jun and Fos family protein expression in human breast cancer: correlation of protein expression and clinicopathological parameters. *European journal of gynaecological oncology* **27**, 345 (2006).
21. X. Ouyang *et al.*, Activator protein-1 transcription factors are associated with progression and recurrence of prostate cancer. *Cancer research* **68**, 2132 (Apr 1, 2008).
22. Y. Shimizu *et al.*, Growth inhibition of non-small cell lung cancer cells by AP-1 blockade using a cJun dominant-negative mutant. *British journal of cancer* **98**, 915 (Mar 11, 2008).
23. E. Passegue, E. F. Wagner, JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. *The EMBO journal* **19**, 2969 (Jun 15, 2000).

24. M. M. Caffarel *et al.*, JunD is involved in the antiproliferative effect of Delta9-tetrahydrocannabinol on human breast cancer cells. *Oncogene* **27**, 5033 (Aug 28, 2008).
25. J. Y. Jin, H. Ke, R. P. Hall, J. Y. Zhang, c-Jun promotes whereas JunB inhibits epidermal neoplasia. *The Journal of investigative dermatology* **131**, 1149 (May, 2011).
26. A. J. Whitmarsh, R. J. Davis, Regulation of transcription factor function by phosphorylation. *Cellular and molecular life sciences : CMLS* **57**, 1172 (Aug, 2000).
27. L. Bakiri, D. Lallemand, E. Bossy-Wetzel, M. Yaniv, Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *The EMBO journal* **19**, 2056 (May 2, 2000).
28. E. Stepniak *et al.*, c-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPK activity. *Genes & development* **20**, 2306 (Aug 15, 2006).
29. A. Gallo *et al.*, Menin uncouples Elk-1, JunD and c-Jun phosphorylation from MAP kinase activation. *Oncogene* **21**, 6434 (Sep 19, 2002).
30. J. B. Weitzman, L. Fiette, K. Matsuo, M. Yaniv, JunD protects cells from p53-dependent senescence and apoptosis. *Molecular cell* **6**, 1109 (Nov, 2000).
31. E. Passegue, W. Jochum, A. Behrens, R. Ricci, E. F. Wagner, JunB can substitute for Jun in mouse development and cell proliferation. *Nature genetics* **30**, 158 (Feb, 2002).
32. E. Tulchinsky, Fos family members: regulation, structure and role in oncogenic transformation. *Histology and histopathology* **15**, 921 (Jul, 2000).
33. I. W. Mak, R. E. Turcotte, S. Popovic, G. Singh, M. Ghert, AP-1 as a Regulator of MMP-13 in the Stromal Cell of Giant Cell Tumor of Bone. *Biochemistry research international* **2011**, 164197 (2011).
34. A. Arai *et al.*, Fos plays an essential role in the upregulation of RANK expression in osteoclast precursors within the bone microenvironment. *Journal of cell science* **125**, 2910 (Jun 15, 2012).
35. C. Nemos, R. Delage-Mourroux, M. Jouvenot, P. Adami, Onset of direct 17-beta estradiol effects on proliferation and c-fos expression during oncogenesis of endometrial glandular epithelial cells. *Experimental cell research* **296**, 109 (Jun 10, 2004).
36. C. Dube *et al.*, The nuclear receptors SF1 and LRH1 are expressed in endometrial cancer cells and regulate steroidogenic gene transcription by cooperating with AP-1 factors. *Cancer letters* **275**, 127 (Mar 8, 2009).

37. L. Bai *et al.*, ERK1/2 promoted proliferation and inhibited apoptosis of human cervical cancer cells and regulated the expression of c-Fos and c-Jun proteins. *Medical oncology* **32**, 57 (Mar, 2015).
38. S. Xiao *et al.*, Fra-1 is downregulated in cervical cancer tissues and promotes cervical cancer cell apoptosis by p53 signaling pathway in vitro. *International journal of oncology* **46**, 1677 (Apr, 2015).
39. S. Hein *et al.*, Expression of Jun and Fos proteins in ovarian tumors of different malignant potential and in ovarian cancer cell lines. *Oncology reports* **22**, 177 (Jul, 2009).
40. L. Oliveira-Ferrer *et al.*, c-FOS suppresses ovarian cancer progression by changing adhesion. *British journal of cancer* **110**, 753 (Feb 4, 2014).
41. M. E. Ramos-Nino, S. R. Blumen, H. Pass, B. T. Mossman, Fra-1 governs cell migration via modulation of CD44 expression in human mesotheliomas. *Molecular cancer* **6**, 81 (2007).
42. M. E. Ramos-Nino *et al.*, HGF mediates cell proliferation of human mesothelioma cells through a PI3K/MEK5/Fra-1 pathway. *American journal of respiratory cell and molecular biology* **38**, 209 (Feb, 2008).
43. J. Ming, G. Jiang, Q. Zhang, X. Qiu, E. Wang, Interleukin-7 up-regulates cyclin D1 via activator protein-1 to promote proliferation of cell in lung cancer. *Cancer immunology, immunotherapy : CII* **61**, 79 (Jan, 2012).
44. J. Wang *et al.*, FOSL2 positively regulates TGF-beta1 signalling in non-small cell lung cancer. *PloS one* **9**, e112150 (2014).
45. Y. Kou, S. Zhang, X. Chen, S. Hu, Gene expression profile analysis of colorectal cancer to investigate potential mechanisms using bioinformatics. *OncoTargets and therapy* **8**, 745 (2015).
46. H. Liu *et al.*, Aberrantly expressed Fra-1 by IL-6/STAT3 transactivation promotes colorectal cancer aggressiveness through epithelial-mesenchymal transition. *Carcinogenesis* **36**, 459 (Apr, 2015).
47. J. Guinea-Viniegra *et al.*, Differentiation-induced skin cancer suppression by FOS, p53, and TACE/ADAM17. *The Journal of clinical investigation* **122**, 2898 (Aug, 2012).
48. E. M. Briso *et al.*, Inflammation-mediated skin tumorigenesis induced by epidermal c-Fos. *Genes & development* **27**, 1959 (Sep 15, 2013).

49. D. C. Kang, M. Motwani, P. B. Fisher, Role of the transcription factor AP-1 in melanoma differentiation (review). *International journal of oncology* **13**, 1117 (Dec, 1998).
50. K. Mallikarjuna, V. Pushparaj, J. Biswas, S. Krishnakumar, Expression of insulin-like growth factor receptor (IGF-1R), c-Fos, and c-Jun in uveal melanoma: an immunohistochemical study. *Current eye research* **31**, 875 (Oct, 2006).
51. Y. H. Kim *et al.*, Fra-1 expression in malignant and benign thyroid tumor. *The Korean journal of internal medicine* **16**, 93 (Jun, 2001).
52. A. Kataki *et al.*, P53 and C-FOS overexpression in patients with thyroid cancer: an immunohistochemical study. *Neoplasma* **50**, 26 (2003).
53. S. Hussain *et al.*, Transcription factor AP-1 in esophageal squamous cell carcinoma: alterations in activity and expression during human Papillomavirus infection. *BMC cancer* **9**, 329 (2009).
54. A. Usui *et al.*, The molecular role of Fra-1 and its prognostic significance in human esophageal squamous cell carcinoma. *Cancer* **118**, 3387 (Jul 1, 2012).
55. T. Watanabe *et al.*, Protein kinase R modulates c-Fos and c-Jun signaling to promote proliferation of hepatocellular carcinoma with hepatitis C virus infection. *PloS one* **8**, e67750 (2013).
56. P. Wang, L. Ouyang, L. Zheng, Z. Wang, Identifying hepatocellular carcinoma-related genes and pathways by system biology analysis. *Irish journal of medical science* **184**, 357 (Jun, 2015).
57. X. Liu *et al.*, Identification of therapeutic targets for breast cancer using biological informatics methods. *Molecular medicine reports* **12**, 1789 (Aug, 2015).
58. D. Roy *et al.*, Integrated Bioinformatics, Environmental Epidemiologic and Genomic Approaches to Identify Environmental and Molecular Links between Endometriosis and Breast Cancer. *International journal of molecular sciences* **16**, 25285 (2015).
59. J. Basbous, I. Jariel-Encontre, T. Gomard, G. Bossis, M. Piechaczyk, Ubiquitin-independent- versus ubiquitin-dependent proteasomal degradation of the c-Fos and Fra-1 transcription factors: is there a unique answer? *Biochimie* **90**, 296 (Feb, 2008).
60. T. Tanos *et al.*, Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. *The Journal of biological chemistry* **280**, 18842 (May 13, 2005).
61. E. Shaulian, M. Karin, AP-1 as a regulator of cell life and death. *Nature cell biology* **4**, E131 (May, 2002).

62. A. Wang *et al.*, Transcription factor complex AP-1 mediates inflammation initiated by Chlamydia pneumoniae infection. *Cellular microbiology* **15**, 779 (May, 2013).
63. L. Chang, M. Karin, Mammalian MAP kinase signalling cascades. *Nature* **410**, 37 (Mar 1, 2001).
64. M. C. Gruda, K. Kovary, R. Metz, R. Bravo, Regulation of Fra-1 and Fra-2 phosphorylation differs during the cell cycle of fibroblasts and phosphorylation in vitro by MAP kinase affects DNA binding activity. *Oncogene* **9**, 2537 (Sep, 1994).
65. F. Mechta, D. Lallemand, C. M. Pfarr, M. Yaniv, Transformation by ras modifies AP1 composition and activity. *Oncogene* **14**, 837 (Feb 20, 1997).
66. B. B. Aggarwal, Signalling pathways of the TNF superfamily: a double-edged sword. *Nature reviews. Immunology* **3**, 745 (Sep, 2003).
67. H. van Dam *et al.*, Autocrine growth and anchorage independence: two complementing Jun-controlled genetic programs of cellular transformation. *Genes & development* **12**, 1227 (Apr 15, 1998).
68. V. Ramirez-Carrozzi, T. Kerppola, Asymmetric recognition of nonconsensus AP-1 sites by Fos-Jun and Jun-Jun influences transcriptional cooperativity with NFAT1. *Molecular and cellular biology* **23**, 1737 (Mar, 2003).
69. A. Sunters, D. P. Thomas, W. A. Yeudall, A. E. Grigoriadis, Accelerated cell cycle progression in osteoblasts overexpressing the c-fos proto-oncogene: induction of cyclin A and enhanced CDK2 activity. *The Journal of biological chemistry* **279**, 9882 (Mar 12, 2004).
70. E. Passegue, W. Jochum, M. Schorpp-Kistner, U. Mohle-Steinlein, E. F. Wagner, Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking junB expression in the myeloid lineage. *Cell* **104**, 21 (Jan 12, 2001).
71. T. Kanno *et al.*, JunB promotes cell invasion and angiogenesis in VHL-defective renal cell carcinoma. *Oncogene* **31**, 3098 (Jun 21, 2012).
72. M. M. Vleugel, A. E. Greijer, R. Bos, E. van der Wall, P. J. van Diest, c-Jun activation is associated with proliferation and angiogenesis in invasive breast cancer. *Human pathology* **37**, 668 (Jun, 2006).
73. L. Blau *et al.*, Aberrant expression of c-Jun in glioblastoma by internal ribosome entry site (IRES)-mediated translational activation. *Proceedings of the National Academy of Sciences of the United States of America* **109**, E2875 (Oct 16, 2012).

74. V. Leventaki *et al.*, c-JUN N-terminal kinase (JNK) is activated and contributes to tumor cell proliferation in classical Hodgkin lymphoma. *Human pathology* **45**, 565 (Mar, 2014).
75. A. Behrens, M. Sibilia, E. F. Wagner, Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nature genetics* **21**, 326 (Mar, 1999).
76. M. Schreiber *et al.*, Control of cell cycle progression by c-Jun is p53 dependent. *Genes & development* **13**, 607 (Mar 1, 1999).
77. T. Zou *et al.*, JunD enhances miR-29b levels transcriptionally and posttranscriptionally to inhibit proliferation of intestinal epithelial cells. *American journal of physiology. Cell physiology* **308**, C813 (May 15, 2015).
78. S. Finch, E. Joseloff, T. Bowden, JunB negatively regulates AP-1 activity and cell proliferation of malignant mouse keratinocytes. *Journal of cancer research and clinical oncology* **128**, 3 (Jan, 2002).
79. K. Podar *et al.*, Up-regulation of c-Jun inhibits proliferation and induces apoptosis via caspase-triggered c-Abl cleavage in human multiple myeloma. *Cancer research* **67**, 1680 (Feb 15, 2007).
80. Y. Y. Yeap *et al.*, c-Jun N-terminal kinase/c-Jun inhibits fibroblast proliferation by negatively regulating the levels of stathmin/oncoprotein 18. *The Biochemical journal* **430**, 345 (Sep 1, 2010).
81. F. Ikeda *et al.*, JNK/c-Jun signaling mediates an anti-apoptotic effect of RANKL in osteoclasts. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **23**, 907 (Jun, 2008).
82. A. Papoudou-Bai *et al.*, The expression levels of JunB, JunD and p-c-Jun are positively correlated with tumor cell proliferation in diffuse large B-cell lymphomas. *Leukemia & lymphoma*, 1 (May 12, 2015).
83. K. Milde-Langosch, H. Kappes, S. Riethdorf, T. Loning, A. M. Bamberger, FosB is highly expressed in normal mammary epithelia, but down-regulated in poorly differentiated breast carcinomas. *Breast cancer research and treatment* **77**, 265 (Feb, 2003).
84. A. C. Obenauf *et al.*, Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature* **520**, 368 (Apr 16, 2015).

85. R. Gao, D. K. Ball, B. Perbal, D. R. Brigstock, Connective tissue growth factor induces c-fos gene activation and cell proliferation through p44/42 MAP kinase in primary rat hepatic stellate cells. *Journal of hepatology* **40**, 431 (Mar, 2004).
86. T. Nakayama *et al.*, Aberrant expression of Fra-2 promotes CCR4 expression and cell proliferation in adult T-cell leukemia. *Oncogene* **27**, 3221 (May 22, 2008).
87. E. M. Hurt *et al.*, Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. *Cancer cell* **5**, 191 (Feb, 2004).
88. J. A. Recio, G. Merlino, Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. *Oncogene* **21**, 1000 (Feb 7, 2002).
89. D. R. Echlin, H. J. Tae, N. Mitin, E. J. Taparowsky, B-ATF functions as a negative regulator of AP-1 mediated transcription and blocks cellular transformation by Ras and Fos. *Oncogene* **19**, 1752 (Mar 30, 2000).
90. N. Wang *et al.*, c-Jun triggers apoptosis in human vascular endothelial cells. *Circulation research* **85**, 387 (Sep 3, 1999).
91. C. A. Connor, M. Adriaens, R. Pierini, I. T. Johnson, N. J. Belshaw, Procyanidin induces apoptosis of esophageal adenocarcinoma cells via JNK activation of c-Jun. *Nutrition and cancer* **66**, 335 (2014).
92. D. Kumar *et al.*, Nitrogen mustard exposure of murine skin induces DNA damage, oxidative stress and activation of MAPK/Akt-AP1 pathway leading to induction of inflammatory and proteolytic mediators. *Toxicology letters* **235**, 161 (Jun 15, 2015).
93. X. Zhang *et al.*, c-Fos as a proapoptotic agent in TRAIL-induced apoptosis in prostate cancer cells. *Cancer research* **67**, 9425 (Oct 1, 2007).
94. A. Muscella *et al.*, Anti-apoptotic effects of protein kinase C-delta and c-fos in cisplatin-treated thyroid cells. *British journal of pharmacology* **156**, 751 (Mar, 2009).
95. S. Nakagomi, Y. Suzuki, K. Namikawa, S. Kiryu-Seo, H. Kiyama, Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**, 5187 (Jun 15, 2003).
96. B. W. Ozanne, H. J. Spence, L. C. McGarry, R. F. Hennigan, Transcription factors control invasion: AP-1 the first among equals. *Oncogene* **26**, 1 (Jan 4, 2007).

97. L. Bakiri *et al.*, Fra-1/AP-1 induces EMT in mammary epithelial cells by modulating Zeb1/2 and TGFbeta expression. *Cell death and differentiation* **22**, 336 (Feb, 2015).
98. J. Gao, Q. Yan, J. Wang, S. Liu, X. Yang, Epithelial-to-mesenchymal transition induced by TGF-beta1 is mediated by AP1-dependent EpCAM expression in MCF-7 cells. *Journal of cellular physiology* **230**, 775 (Apr, 2015).
99. E. Y. Yi, S. Y. Park, S. Y. Jung, W. J. Jang, Y. J. Kim, Mitochondrial dysfunction induces EMT through the TGF-beta/Smad/Snail signaling pathway in Hep3B hepatocellular carcinoma cells. *International journal of oncology* **47**, 1845 (Nov, 2015).
100. E. H. Nam, Y. Lee, B. Moon, J. W. Lee, S. Kim, Twist1 and AP-1 cooperatively upregulate integrin alpha5 expression to induce invasion and the epithelial-mesenchymal transition. *Carcinogenesis* **36**, 327 (Mar, 2015).
101. G. Zhang *et al.*, Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents. *Journal of the National Cancer Institute* **96**, 683 (May 5, 2004).
102. J. Ming, Q. Zhang, X. Qiu, E. Wang, Interleukin 7/interleukin 7 receptor induce c-Fos/c-Jun-dependent vascular endothelial growth factor-D up-regulation: a mechanism of lymphangiogenesis in lung cancer. *European journal of cancer (Oxford, England : 1990)* **45**, 866 (Mar, 2009).
103. F. Galvagni, M. Orlandini, S. Oliviero, Role of the AP-1 transcription factor FOSL1 in endothelial cells adhesion and migration. *Cell adhesion & migration* **7**, 408 (Sep-Oct, 2013).
104. W. Jochum, E. Passegue, E. F. Wagner, AP-1 in mouse development and tumorigenesis. *Oncogene* **20**, 2401 (Apr 30, 2001).
105. R. Eferl *et al.*, Functions of c-Jun in liver and heart development. *The Journal of cell biology* **145**, 1049 (May 31, 1999).
106. M. Schorpp-Kistner, Z. Q. Wang, P. Angel, E. F. Wagner, JunB is essential for mammalian placentation. *The EMBO journal* **18**, 934 (Feb 15, 1999).
107. M. Schreiber *et al.*, Placental vascularisation requires the AP-1 component fra1. *Development (Cambridge, England)* **127**, 4937 (Nov, 2000).
108. Z. Q. Wang *et al.*, Bone and haematopoietic defects in mice lacking c-fos. *Nature* **360**, 741 (Dec 24-31, 1992).
109. P. J. Shiromani *et al.*, Sleep and wakefulness in c-fos and fos B gene knockout mice. *Brain research. Molecular brain research* **80**, 75 (Aug 14, 2000).

110. J. R. Brown, H. Ye, R. T. Bronson, P. Dikkes, M. E. Greenberg, A defect in nurturing in mice lacking the immediate early gene fosB. *Cell* **86**, 297 (Jul 26, 1996).
111. A. Bozec *et al.*, Fra-2/AP-1 controls bone formation by regulating osteoblast differentiation and collagen production. *The Journal of cell biology* **190**, 1093 (Sep 20, 2010).
112. F. Paneni *et al.*, Deletion of the activated protein-1 transcription factor JunD induces oxidative stress and accelerates age-related endothelial dysfunction. *Circulation* **127**, 1229 (Mar 19, 2013).
113. K. McPherson, C. M. Steel, J. M. Dixon, ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *Bmj* **321**, 624 (Sep 9, 2000).
114. N. F. Boyd *et al.*, Mammographic density and the risk and detection of breast cancer. *The New England journal of medicine* **356**, 227 (Jan 18, 2007).
115. J. Ferlay *et al.*, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer. Journal international du cancer* **136**, E359 (Mar 1, 2015).
116. . (October 7, 2015).
117. R. Wesolowski, B. Ramaswamy, Gene expression profiling: changing face of breast cancer classification and management. *Gene expression* **15**, 105 (2011).
118. O. Yersal, S. Barutca, Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World journal of clinical oncology* **5**, 412 (Aug 10, 2014).
119. M. I. Nounou *et al.*, Breast Cancer: Conventional Diagnosis and Treatment Modalities and Recent Patents and Technologies. *Breast cancer : basic and clinical research* **9**, 17 (2015).
120. L. A. Carey *et al.*, Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *Jama* **295**, 2492 (Jun 7, 2006).
121. F. Heitz *et al.*, Triple-negative and HER2-overexpressing breast cancers exhibit an elevated risk and an earlier occurrence of cerebral metastases. *European journal of cancer (Oxford, England : 1990)* **45**, 2792 (Nov, 2009).
122. J. I. Herschkowitz *et al.*, Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome biology* **8**, R76 (2007).
123. F. Podo *et al.*, Triple-negative breast cancer: present challenges and new perspectives. *Molecular oncology* **4**, 209 (Jun, 2010).

124. B. D. Lehmann, J. A. Pietenpol, Identification and use of biomarkers in treatment strategies for triple-negative breast cancer subtypes. *The Journal of pathology* **232**, 142 (Jan, 2014).
125. R. Schmadeka, B. E. Harmon, M. Singh, Triple-negative breast carcinoma: current and emerging concepts. *American journal of clinical pathology* **141**, 462 (Apr, 2014).
126. F. Le Du *et al.*, Is the future of personalized therapy in triple-negative breast cancer based on molecular subtype? *Oncotarget* **6**, 12890 (May 30, 2015).
127. I. Kallel, M. Rebai, A. Khabir, A. Rebai, What common biomarkers characterize a triple-negative profile in breast cancer? *Pathologie-biologie* **63**, 224 (Sep, 2015).
128. A. Papa *et al.*, Triple-negative breast cancer: investigating potential molecular therapeutic target. *Expert opinion on therapeutic targets* **19**, 55 (Jan, 2015).
129. C. Williams, C. Y. Lin, Oestrogen receptors in breast cancer: basic mechanisms and clinical implications. *Ecancermedicalscience* **7**, 370 (2013).
130. C. Williams, K. Edvardsson, S. A. Lewandowski, A. Strom, J. A. Gustafsson, A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells. *Oncogene* **27**, 1019 (Feb 7, 2008).
131. C. Lattrich *et al.*, Effects of a combined treatment with tamoxifen and estrogen receptor beta agonists on human breast cancer cell lines. *Archives of gynecology and obstetrics* **289**, 163 (Jan, 2014).
132. H. J. Chan, K. Petrossian, S. Chen, Structural and functional characterization of aromatase, estrogen receptor, and their genes in endocrine-responsive and -resistant breast cancer cells. *The Journal of steroid biochemistry and molecular biology*, (Aug 13, 2015).
133. Y. Liu *et al.*, AP-1 blockade in breast cancer cells causes cell cycle arrest by suppressing G1 cyclin expression and reducing cyclin-dependent kinase activity. *Oncogene* **23**, 8238 (Oct 28, 2004).
134. C. Lu *et al.*, cFos is critical for MCF-7 breast cancer cell growth. *Oncogene* **24**, 6516 (Sep 29, 2005).
135. L. M. Smith *et al.*, cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. *Oncogene* **18**, 6063 (Oct 28, 1999).
136. Y. Zhou *et al.*, Enhanced NF kappa B and AP-1 transcriptional activity associated with antiestrogen resistant breast cancer. *BMC cancer* **7**, 59 (2007).

137. S. Catalano *et al.*, Tamoxifen through GPER upregulates aromatase expression: a novel mechanism sustaining tamoxifen-resistant breast cancer cell growth. *Breast cancer research and treatment* **146**, 273 (Jul, 2014).
138. L. Oliveira-Ferrer *et al.*, Prognostic impact of transcription factor Fra-1 in ER-positive breast cancer: contribution to a metastatic phenotype through modulation of tumor cell adhesive properties. *Journal of cancer research and clinical oncology* **141**, 1715 (Oct, 2015).
139. J. Gao, Q. Yan, J. Wang, S. Liu, X. Yang, Epithelial-to-Mesenchymal Transition Induced by TGF-beta1 Is Mediated by AP1-Dependent EpCAM Expression in MCF-7 Cells. *Journal of cellular physiology*, (Sep 9, 2014).
140. K. Belguise, N. Kersual, F. Galtier, D. Chalbos, FRA-1 expression level regulates proliferation and invasiveness of breast cancer cells. *Oncogene* **24**, 1434 (Feb 17, 2005).
141. R. Dhankhar *et al.*, Advances in novel drug delivery strategies for breast cancer therapy. *Artificial cells, blood substitutes, and immobilization biotechnology* **38**, 230 (Oct, 2010).
142. B. Yeo, N. C. Turner, A. Jones, An update on the medical management of breast cancer. *Bmj* **348**, g3608 (2014).
143. P. D. Grivas, H. Kiaris, A. G. Papavassiliou, Tackling transcription factors: challenges in antitumor therapy. *Trends in molecular medicine* **17**, 537 (Oct, 2011).
144. S. J. Johnston, J. S. Carroll, Transcription factors and chromatin proteins as therapeutic targets in cancer. *Biochimica et biophysica acta* **1855**, 183 (Apr, 2015).
145. L. M. Coussens, Z. Werb, Inflammation and cancer. *Nature* **420**, 860 (Dec 19-26, 2002).
146. S. P. Hussain, C. C. Harris, Inflammation and cancer: an ancient link with novel potentials. *International journal of cancer. Journal international du cancer* **121**, 2373 (Dec 1, 2007).
147. C. D. Morrison, J. G. Parvani, W. P. Schiemann, The relevance of the TGF-beta Paradox to EMT-MET programs. *Cancer letters* **341**, 30 (Nov 28, 2013).
148. D. Wang, R. N. DuBois, Immunosuppression associated with chronic inflammation in the tumor microenvironment. *Carcinogenesis* **36**, 1085 (Oct, 2015).
149. E. A. Carswell *et al.*, An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 3666 (Sep, 1975).

150. L. J. Old, Tumor necrosis factor (TNF). *Science (New York, N.Y.)* **230**, 630 (Nov 8, 1985).
151. F. Balkwill, Tumour necrosis factor and cancer. *Nature reviews. Cancer* **9**, 361 (May, 2009).
152. E. A. Hamed, M. M. Zakhary, D. W. Maximous, Apoptosis, angiogenesis, inflammation, and oxidative stress: basic interactions in patients with early and metastatic breast cancer. *Journal of cancer research and clinical oncology* **138**, 999 (Jun, 2012).
153. D. Faustman, M. Davis, TNF receptor 2 pathway: drug target for autoimmune diseases. *Nature reviews. Drug discovery* **9**, 482 (Jun, 2010).
154. P. J. Naude, J. A. den Boer, P. G. Luiten, U. L. Eisel, Tumor necrosis factor receptor cross-talk. *The FEBS journal* **278**, 888 (Apr, 2011).
155. A. Devin *et al.*, The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity* **12**, 419 (Apr, 2000).
156. M. Schena, D. Shalon, R. W. Davis, P. O. Brown, Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science (New York, N.Y.)* **270**, 467 (Oct 20, 1995).
157. M. Schena *et al.*, Microarrays: biotechnology's discovery platform for functional genomics. *Trends in biotechnology* **16**, 301 (Jul, 1998).
158. A. Dou, Z. Wang, J. Zhao, J. Liu, C. Zheng, Identification of therapeutic target genes with DNA microarray in multiple myeloma cell line treated by IKKbeta/NF-kappaB inhibitor. *Acta cirurgica brasileira / Sociedade Brasileira para Desenvolvimento Pesquisa em Cirurgia* **29**, 696 (Nov, 2014).
159. H. L. Huang *et al.*, Discovery of prognostic biomarkers for predicting lung cancer metastasis using microarray and survival data. *BMC bioinformatics* **16**, 54 (2015).
160. T. Matsumura *et al.*, Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. *British journal of cancer* **113**, 275 (Jul 14, 2015).
161. D. S. Gilmour, J. T. Lis, Detecting protein-DNA interactions in vivo: distribution of RNA polymerase on specific bacterial genes. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4275 (Jul, 1984).
162. D. S. Gilmour, J. T. Lis, In vivo interactions of RNA polymerase II with genes of Drosophila melanogaster. *Molecular and cellular biology* **5**, 2009 (Aug, 1985).

163. D. S. Gilmour, J. T. Lis, RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in Drosophila melanogaster cells. *Molecular and cellular biology* **6**, 3984 (Nov, 1986).
164. D. S. Gilmour, J. T. Lis, Protein-DNA cross-linking reveals dramatic variation in RNA polymerase II density on different histone repeats of Drosophila melanogaster. *Molecular and cellular biology* **7**, 3341 (Sep, 1987).
165. G. Robertson *et al.*, Genome-wide profiles of STAT1 DNA association using chromatin immunoprecipitation and massively parallel sequencing. *Nature methods* **4**, 651 (Aug, 2007).
166. K. R. Chng, E. Cheung, Sequencing the transcriptional network of androgen receptor in prostate cancer. *Cancer letters* **340**, 254 (Nov 1, 2013).
167. T. Lu *et al.*, Role of lysine methylation of NF-kappaB in differential gene regulation. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13510 (Aug 13, 2013).
168. V. K. Tiwari *et al.*, A chromatin-modifying function of JNK during stem cell differentiation. *Nature genetics* **44**, 94 (Jan, 2012).
169. X. Mao *et al.*, Amplification and overexpression of JUNB is associated with primary cutaneous T-cell lymphomas. *Blood* **101**, 1513 (Feb 15, 2003).
170. X. Mao *et al.*, A genomic and expression study of AP-1 in primary cutaneous T-cell lymphoma: evidence for dysregulated expression of JUNB and JUND in MF and SS. *Journal of cutaneous pathology* **35**, 899 (Oct, 2008).
171. S. C. Biddie *et al.*, Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding. *Molecular cell* **43**, 145 (Jul 8, 2011).
172. F. Zanconato *et al.*, Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nature cell biology* **17**, 1218 (Sep, 2015).
173. P. K. Srivastava, R. P. Hull, J. Behmoaras, E. Petretto, T. J. Aitman, JunD/AP1 regulatory network analysis during macrophage activation in a rat model of crescentic glomerulonephritis. *BMC systems biology* **7**, 93 (2013).
174. E. Charafe-Jauffret *et al.*, Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* **25**, 2273 (Apr 6, 2006).
175. J. Kao *et al.*, Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PloS one* **4**, e6146 (2009).

176. C. K. Osborne, K. Hobbs, J. M. Trent, Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast cancer research and treatment* **9**, 111 (1987).
177. H. Cao, J. M. Shockey, Comparison of TaqMan and SYBR Green qPCR methods for quantitative gene expression in tung tree tissues. *Journal of agricultural and food chemistry* **60**, 12296 (Dec 19, 2012).
178. J. S. Carroll *et al.*, Genome-wide analysis of estrogen receptor binding sites. *Nature genetics* **38**, 1289 (Nov, 2006).
179. Y. Liu *et al.*, The genome landscape of ERalpha- and ERbeta-binding DNA regions. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 2604 (Feb 19, 2008).
180. W. Wang, L. Dong, B. Saville, S. Safe, Transcriptional activation of E2F1 gene expression by 17beta-estradiol in MCF-7 cells is regulated by NF-Y-Sp1/estrogen receptor interactions. *Molecular endocrinology (Baltimore, Md.)* **13**, 1373 (Aug, 1999).
181. D. K. Dimova, N. J. Dyson, The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24**, 2810 (Apr 18, 2005).
182. S. Chung *et al.*, Overexpressing PKIB in prostate cancer promotes its aggressiveness by linking between PKA and Akt pathways. *Oncogene* **28**, 2849 (Aug 13, 2009).
183. S. K. Pal, B. H. Childs, M. Pegram, Triple negative breast cancer: unmet medical needs. *Breast cancer research and treatment* **125**, 627 (Feb, 2011).
184. R. M. Neve *et al.*, A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer cell* **10**, 515 (Dec, 2006).
185. V. Walia *et al.*, hCLCA2 Is a p53-Inducible Inhibitor of Breast Cancer Cell Proliferation. *Cancer research* **69**, 6624 (Aug 15, 2009).
186. Y. Sasaki *et al.*, CLCA2, a target of the p53 family, negatively regulates cancer cell migration and invasion. *Cancer biology & therapy* **13**, 1512 (Dec, 2012).
187. J. Comijn *et al.*, The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Molecular cell* **7**, 1267 (Jun, 2001).
188. L. J. Talbot, S. D. Bhattacharya, P. C. Kuo, Epithelial-mesenchymal transition, the tumor microenvironment, and metastatic behavior of epithelial malignancies. *International journal of biochemistry and molecular biology* **3**, 117 (2012).
189. E. Lopez-Knowles *et al.*, PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. *International journal of cancer. Journal international du cancer* **126**, 1121 (Mar 1, 2010).

190. C. Bartholomeusz *et al.*, High ERK protein expression levels correlate with shorter survival in triple-negative breast cancer patients. *The oncologist* **17**, 766 (2012).
191. G. Soria *et al.*, Inflammatory mediators in breast cancer: coordinated expression of TNFalpha & IL-1beta with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition. *BMC cancer* **11**, 130 (2011).
192. S. J. Cho *et al.*, Ninjurin1, a target of p53, regulates p53 expression and p53-dependent cell survival, senescence, and radiation-induced mortality. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 9362 (Jun 4, 2013).
193. Y. Wu *et al.*, Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. *Cancer cell* **15**, 416 (May 5, 2009).
194. Y. Ben-Neriah, M. Karin, Inflammation meets cancer, with NF-kappaB as the matchmaker. *Nature immunology* **12**, 715 (Aug, 2011).
195. L. Jurida *et al.*, The Activation of IL-1-Induced Enhancers Depends on TAK1 Kinase Activity and NF-kappaB p65. *Cell reports*, (Feb 4, 2015).
196. N. Ye, Y. Ding, C. Wild, Q. Shen, J. Zhou, Small molecule inhibitors targeting activator protein 1 (AP-1). *Journal of medicinal chemistry* **57**, 6930 (Aug 28, 2014).